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PHOSPHOLIPID-PROTHROMBIN-Ca^{++} COMPLEXES

by

PAUL MACKEY

©

A THESIS

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "PHOSPHOLIPID-PROTHROMBIN-Ca^{++} COMPLEXES" submitted by PAUL MACKEY in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

The nature of the interactions between prothrombin, Ca^{++} and phospholipids has been investigated by nuclear magnetic resonance spectroscopy (NMR). Mixtures of cytochrome c and phospholipids were also examined for comparative purposes.

The NMR spectra of mixtures of phosphatidyl choline (PC) and phosphatidyl serine (PS) containing 0% to 80% PS were obtained. With mixtures containing 40% or more PS, the $-CH_2-$ resonance was split into two components and the $-CH_3$ resonance into three. In both cases, the relative intensity of the upfield component increased with higher PS values. This was interpreted as a phase separation into a mixed PS:PC phase, producing the downfield component, and a PS-rich phase, producing the upfield component.

Addition of cytochrome c or Ca^{++} to pure PC did not broaden the $-\overset{+}{N}(CH_3)_3$, $-CH_2-$ or $-CH_3$ signals. For mixtures of PC with 20%, 40%, 60% and 80% PS, cytochrome c increased the $-\overset{+}{N}(CH_3)_3$ linewidth and decreased the intensity of the downfield components of the $-CH_2-$ and $-CH_3$ resonances. The addition of Ca^{++} was more effective than cytochrome c both in broadening the $-\overset{+}{N}(CH_3)_3$ resonance and in decreasing the downfield components of the $-CH_2-$ and $-CH_3$ signals. The addition of prothrombin to phospholipid-Ca^{++} mixtures produced little further change in the NMR spectra. This supported a model whereby prothrombin and phospholipid

interact principally through Ca^{++} chelation.

Attempts to repeat these experiments with another preparation of PS did not result in the splitting of the $-CH_2-$ and $-CH_3$ resonances. Several possible explanations for this discrepancy between one preparation and another were considered. These included differences in fatty acid composition, amounts of autoxidation products present, purity as judged by thin-layer chromatography, extent of degradation during sonication, efficiency of cavitation and the effect of paramagnetic ions. No completely satisfactory answer was found. Discrepancies in the literature are discussed.

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TABLE OF CONTENTS

	Page
Abstract	iv
Acknowledgements	vi
List of Tables	x
List of Illustrations	xi
List of Abbreviations	xiii
CHAPTER	
I. INTRODUCTION	1
Phospholipids in water	2
Sonication	4
Nuclear magnetic resonance of phospholipids	8
Blood Coagulation: Molecular Characteristics	19
Role of Vitamin K	31
The aim of the research	37
II. MATERIALS AND METHODS	
A. Materials	39
B. Methods	
1. Phosphorus determinations	40
2. Protein determinations	40
3. Phospholipid sample preparations	40
4. Sonication	41
5. Nuclear magnetic resonance	41

TABLE OF CONTENTS (Cont'd.)

CHAPTER		Page
II.	6. Fatty acid analysis	41
	7. Thin-layer chromatography	42
	8. Amino acid analysis	42
	9. Electrophoresis	42
	10. Coagulation assays	42
	11. Ultracentrifuge experiments	43
III.	PROTHROMBIN-Ca^{++}-PHOSPHOLIPID COMPLEXES	
	A. Prothrombin characterization	
	1. Sephadex chromatography	45
	2. Amino acid analysis	45
	3. Ultracentrifuge studies	48
	4. Disc gel electrophoresis	48
	5. Clotting assay	48
	B. Preparation procedures	52
	C. Results	
	1. Choline methyl linewidths	53
	2. Methylene resonance splitting ...	62
	3. Methyl resonance splitting	64
	4. Discussion	73
IV.	A RE-EVALUATION OF THE PHOSPHOLIPID COMPONENT	82
	1. Purity of the PS preparations ...	85
	2. Autoxidation of phosphatidyl serine	85

TABLE OF CONTENTS (Cont'd.)

CHAPTER		Page
IV.	3. Effect of sonication on physical structure	89
	4. Effect of sonication on chemical structure	89
	5. Effect of contaminating paramagnetic ions on linewidths	90
V.	DISCUSSION	
	Structure and properties of Ca^{++} complexes	92
	Ca^{++}-lipid complexes	94
	Phase separations in mixed lipid bilayers	99
	Ca^{++}-protein complexes	103
	Lipid-Ca^{++}-protein complexes	104
	Other model systems	105
	Biological significance of prothrombin-Ca^{++}-lipid complexes	109
	Suggestions for further work	114
BIBLIOGRAPHY		117

LIST OF TABLES

TABLE		Page
I.	Blood Clotting Factor Nomenclature	20
II.	Complex Formation during Prothrombin Activation	30
III.	Amino Acid Composition of Bovine Prothrombin	47
IV.	NMR Linewidths Using the PS-2 Preparation ..	84
V.	PS Purification by DEAE-cellulose acetate chromatography	86
VI.	Fatty acid analysis of phosphatidyl serine preparations from bovine brain white matter by various workers	88
VII.	Association constants (K_a) for Ca^{++}-lipid complexes and comparison with model compounds	95

LIST OF ILLUSTRATIONS

FIGURE		Page
1.	Phospholipids in water	3
2.	Prothrombin activation	28
3.	Calibration curve for one-stage prothrombin assay	44
4.	Sephadex G-200 chromatography of prothrombin ..	46
5.	Sedimentation velocity ultracentrifugation of prothrombin	49
6.	Concentration dependence of the sedimentation coefficient	50
7.	SDS-polyacrylamide gel electrophoresis of prothrombin	51
8.	NMR spectrum of 20% PS:80% PC	55
9.	NMR spectrum of 20% PS:80% PC with cytochrome c	57
10.	NMR spectrum of 20% PS:80% PC with Ca^{++}	59
11.	NMR spectrum of 20% PS:80% PC with Ca^{++} and prothrombin	61
12.	$-\overset{+}{N}(CH_3)_3$ linewidths	63
13.	The $-CH_2-$ region of the NMR spectra of PS:PC mixtures	66
14.	The $-CH_2-$ region of the NMR spectra of PS:PC mixtures with cytochrome c	68

LIST OF ILLUSTRATIONS (Cont'd.)

FIGURE		Page
15.	The $-CH_2-$ region of the NMR spectra of PS:PC mixtures with Ca^{++}	70
16.	The $-CH_2-$ region of the NMR spectra of PS:PC mixtures with Ca^{++} and prothrombin	72
17.	The $-CH_3-$ region of the NMR spectra of PS:PC mixtures with and without cytochrome c	75
18.	The $-CH_3-$ region of the NMR spectra of PS:PC mixtures with Ca^{++} and with or without prothrombin	77
19.	Diagrammatic representation of the proposed interaction between Ca^{++} and PS:PC mixtures	79
20.	Thin-layer chromatography of PS preparations ..	83
21.	Thin-layer chromatographic investigation of the effect of sonication	91
22.	Possible structure for a PS-metal complex	96
23.	A linear polymeric lattice structure for a PS-metal complex	98
24.	Schematic representation of a possible structural unit linking lipid and protein	106
25.	Schematic representation of lipid-protein interactions involved in prothrombin activation	113

LIST OF ABBREVIATIONS

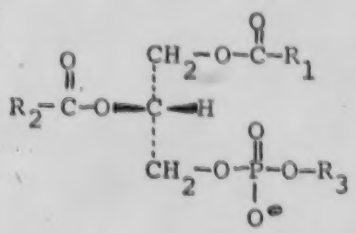
A_{280}	absorbance at a wavelength of 280 nm
CD	circular dichroism
DEAE	diethyl aminoethyl
DFP	diisopropyl phosphorofluoridate
DSC	differential scanning calorimetry
ECL	equivalent chain length
EDTA	ethylene diamine tetraacetic acid
ESR	electron spin resonance
GLC	gas-liquid chromatography
K_a	association constant
M.W.	molecular weight
NE	not examined
NMR	nuclear magnetic resonance
P	phosphorus
PA	phosphatidic acid
PC	phosphatidyl choline or lecithin
PE	phosphatidyl ethanolamine
PG	phosphatidyl glycerol
PI	phosphatidyl inositol
PL	phospholipid
PS	phosphatidyl serine
$S^o_{20,w}$	intrinsic sedimentation coefficient
SDS	sodium dodecyl sulphate

T_1	spin-lattice or longitudinal relaxation time
T_2	spin-spin or transverse relaxation time
T_2^{eff}	effective spin-spin relaxation time
TLC	thin-layer chromatography

CHAPTER I

INTRODUCTION

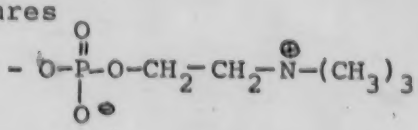
Glycerophospholipids are a class of compounds having the general structure

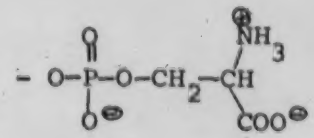


where R_1 and R_2 are hydrocarbon chains

They are structurally derived from phosphatidic acid, the simplest member of the family, where R_3 is hydrogen. Our own work has focussed on two phospholipid classes, 1,2-diacyl-sn-glycero-3-phosphoryl choline (phosphatidyl choline) and 1,2-diacyl-sn-glycero-3-phosphoryl serine (phosphatidyl serine), isolated respectively from egg yolk and beef brain. Each contains a characteristic spectrum of fatty acids (Klenk and Böhm 1951, Hawke 1959, Hanahan et al. 1960, Silver et al. 1963, Graham and Lea 1972).

At neutral pH, phosphatidyl choline and phosphatidyl serine have the following respective charge structures





The two hydrocarbon chains of phospholipids can be arranged parallel to each other while the polar group lies on the opposite side of the glycerol moiety. This separation of polar and non-polar entities is reflected in strongly amphipathic behaviour. Thus, in chloroform, above the critical micellar concentration (11.1 mg/ml at 24°C), dipalmitoyl PC forms micelles, each composed of three molecules intra-molecularly hydrogen-bonded through the polar region (Haque et al. 1972). Three-membered micelles are also formed in methanol while in benzene, the micelles are larger, with 73 molecules at 25°C (Elworthy and McIntosh 1961).

Phospholipids in water

When dispersed in water by shaking, phospholipids form more extensive structures (Bangham 1963, Bangham 1968). Over a wide range of lipid concentration in water, a smectic liquid crystalline phase is present. In the case of phosphatidyl cholines, this consists of repeating concentric bimolecular lamellae of lipid molecules separated by water, as shown in figure 1.

Bovine brain PS, shaken in water, forms a variety of structures dependent on the ionic strength of the medium. This is presumably due to the electrostatic repulsion of the charged polar region (Hauser and Phillips 1973, Atkinson et al. 1974).

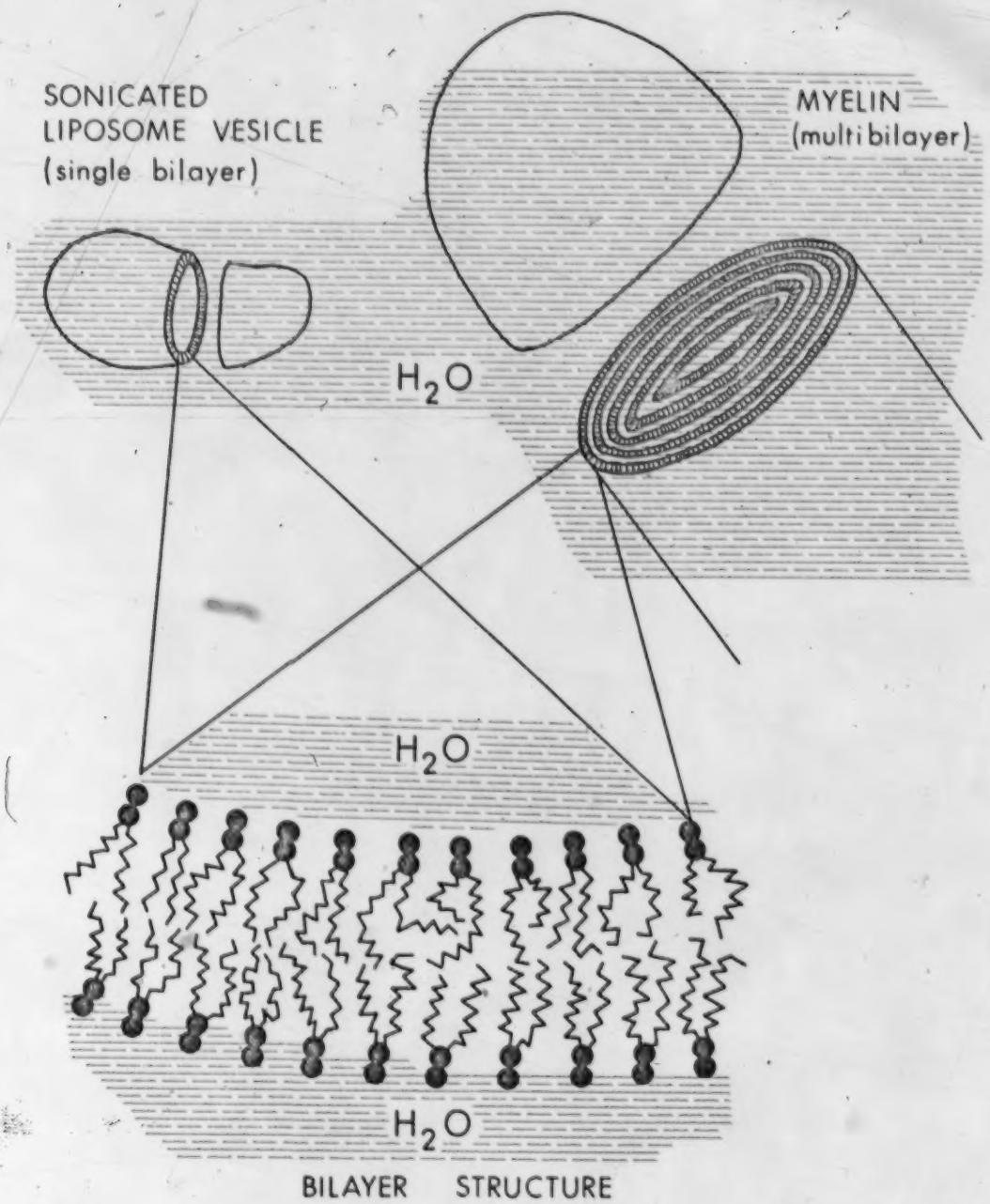


Figure 1. Schematic representation of phospholipid structures formed in an aqueous environment. Solid circles represent polar groups of the phospholipids while zig-zag lines represent the hydrocarbon chains.

The nature of the hydrocarbon chains also influences the structures. At a critical temperature, the transition temperature, chains undergo a phase change from the gel to the liquid-crystalline state. The transition temperature and the entropy change both decrease from dibehenoyl (C_{22}) phosphatidyl choline to the dimyristoyl (C_{14}) analogue (Chapman et al. 1967, Phillips et al. 1969). Similarly, the transition temperature of unsaturated PC is lower than that of saturated PC (Ladbrooke et al. 1968). X-ray studies have shown that unsaturated phospholipids occupy a larger surface area per molecule than saturated ones. These latter assume a more rigid configuration (Engelman 1971).

Sonication

Dispersed phospholipids in water give rise to a very broad NMR signal, approximately 500 cycles wide (Chapman et al. 1968) at 60 MHz, although chemically shifted signals can be observed at 220 MHz (Finer 1972). It is, however, possible to obtain a high resolution spectrum if the phospholipid is sonicated. Ultrasonication produces cavitation of gases dissolved in the suspending liquid (Hughes and Nyborg 1962). The turbid phospholipid suspension becomes opalescent or clear, indicating a breakdown of particle sizes. This is confirmed by light scattering (Attwood and Saunders 1965) and electron microscopy (Chapman et al. 1968).

On the basis of electron microscope measurements, Saunders (1966) suggested that sonication expelled water and folded the fragments into rolled-up laminae as in "Swiss rolls". Johnson et al. (1971) demonstrated that these pictures were due to artifacts of the drying process and that in fact single bilayer spherical liposomes were formed. A diameter of 240 ± 8 Å was indicated by their ultracentrifuge diffusion experiments for 96% egg PC: 4% phosphatidic acid. Huang (1969) found that two fractions, separable by Sepharose 4B chromatography, were always produced by sonication of egg PC. One consisted of large aggregates and the other of a homogeneous population of small vesicles. These latter particles were examined by a number of techniques and the particle diameters found to be 280 ± 10 Å by molecular sieve chromatography, 250 ± 20 Å by freeze-etch electron microscopy, 300 ± 30 Å by negative stain electron microscopy, 200 to 306 Å from particle weight, X-ray data and geometrical considerations, and 228 ± 5 Å by ultracentrifuge diffusion.

Sonication of PS also leads to the formation of single bilayer vesicles. These are smaller than those of PC: 62 to 125 Å as estimated from Sepharose 4B chromatography and 80 to 100 Å from light scattering experiments (Hauser and Phillips 1973, Atkinson et al. 1974). Consequently, PS vesicles have a higher curvature.

X-ray studies by Chapman et al. (1968) have shown that the bilayer repeat spacing of 66 Å is still present after sonication. ESR hydrophobic probe studies have also confirmed the retention of the bilayer structure (Finer et al. 1972).

Hauser (1971) and Hauser and Irons (1972) pointed out that ultrasonication may result in oxidation of double bonds or the cleavage of covalent bonds. In their investigation, oxidation was avoided by maintaining the sample under nitrogen and in an ice bath during sonication. There was, nevertheless, some degradation of the PC to lysolecithin (3.5 % by weight after high intensity treatment for 20 minutes and no increase subsequently), fatty acids, choline, glycerylphosphoryl choline and phosphorylcholine (linear increase in amounts with time of sonication). At lower intensities, no degradation was observed before 40 minutes. They reported that sonication of pure PC gives a distribution of vesicle sizes and that the vesicles tend to aggregate. However, the addition of lysolecithin stabilized the preparation and rendered it homogeneous with respect to size.

In contrast, Huang and Charlton (1972) were unable to detect any degradation even after two and a half hours of sonication. They also maintained the sample at 2°C under nitrogen and found that the sonicated vesicles were not homogeneous.

Marsh et al. (1972) have partially separated large incompletely fragmented material from small vesicles produced during sonication by Sepharose 4B chromatography. These vesicles were shown by negative stain electron microscopy to be composed of single bilayers. By ultracentrifugation, the vesicle fraction was judged to be homogeneous but the measurement of the spin probe order parameter using a nitroxide derivative of 5-ketc stearic acid incorporated into the vesicles showed that the latter are not homogeneous because of contamination by the large aggregates. The hydrocarbon regions of the vesicles were slightly more fluid than those of the large aggregates. They concluded that though the effect of sonication is mainly at a macroscopic rather than microscopic level, there is some difference in molecular packing.

Hauser and Irons (1972) argued for no change in the hydrocarbon chain packing since the electron spin correlation time of a nitroxide derivative of methyl oleate incorporated into PC bilayers remained unchanged after sonication. Using a nitroxide derivative of a substituted piperidone, Barratt et al. (1969) had also observed no ESR spectral differences before and after sonication. Sheetz and Chan (1972) are convinced that the simple geometry of the small vesicles with their greater curvature dictates an altered packing. Their dilatometry measurements showed the apparent molal

volume of dipalmitoyl phosphatidyl choline to be larger in vesicles than in bilayers below the transition temperature. However, above the transition temperature, the apparent molal volumes are identical within experimental error.

Finer et al. (1972) followed the progress of sonication by analytical gel filtration of the products and found the kinetics of formation of the vesicles to be second order. They postulated a mechanism whereby the large structures are broken down to small fragments, consisting of a few PC molecules, which then aggregate to form vesicles. The growth of the small vesicle peak on Sepharose 4B chromatography paralleled the growth of the high resolution NMR signal.

Nuclear magnetic resonance of phospholipids

The origin of the high resolution spectrum produced by sonication is a matter of controversy and requires consideration of the possible nuclear relaxation mechanisms involved both in unsonicated and sonicated phospholipids. In order to interpret NMR line broadening or narrowing in terms of decreased or increased motion, it is necessary to verify that the linewidth does reflect dipole-dipole interactions which are influenced by the closeness of packing of the molecules.

Relaxation mechanisms are characterized by a spin-lattice or longitudinal relaxation time (T_1) and a spin-spin or transverse relaxation time (T_2). The former

is related to the distribution of nuclei between spin states while the latter describes the rate of loss of coherence in the x-y plane. We shall consider here only proton relaxation mechanisms unless otherwise stated.

Penkett et al. (1968) reported the existence of a single T_1 for all $-CH_2-$ signals of a viscous PC-water mixture. The linewidth was observed to vary linearly with the magnetic field from 40 to 100 MHz and it was argued on this basis that other relaxation mechanisms, such as magnetic anisotropies, are operative besides dipole-dipole interactions. However, chemical shift anisotropies and those due to the anisotropic motion of lipids in lamellae have too small an effect to account for the results.

Kaufman et al. (1970) investigated lipids extracted from erythrocytes and deduced the presence of magnetic field gradients from Carr-Purcell $180-\tau-90$ pulse results. Molecular diffusion through these gradients was proposed as one mechanism for a similar anisotropic system, dimethyl dodecylamine oxide - water by Hansen and Lawson (1970).

On the other hand, the field independence of T_2^{eff} of another smectic system, sodium caprylate-decanol-water from 8 to 90 MHz (Tiddy 1971) suggested the above interpretation was incorrect. In addition, no dependence of T_2^{eff} from 16 to 60 MHz for unsonicated egg PC (Chan 1971)

and from 10 to 40 MHz for egg PC, dipalmitoyl PC, or potassium laurate was observed (Oldfield et al. 1971). These last workers found dipolar echoes from 90 -τ - 90 pulse sequences and were thus able to state that the spin-spin relaxation is predominantly due to dipolar interactions. They could not reproduce the results of Kaufman et al. (1970). They further argued that the pulse spacings in Hansen and Lawson's (1970) spin-echo experiments on dimethyl dodecylamine oxide - water were too close to the T_2^{eff} and that these conditions could cause a line narrowing effect. In their work, only a single spin-lattice relaxation time was found.

Finer et al. (1972a) confirmed the dipolar nature of the relaxation mechanism by analyzing a sample oriented at 54°44' to the field (the magic angle). The methylene linewidths increased with field strengths from 60 MHz up to 300 MHz. The linewidth dependence on field strength does not necessarily imply non-dipolar contributions because this behaviour corresponds to that observed with solid rubber samples where it is known that the linewidth originates from the incomplete motional averaging of dipolar interactions.

Chan et al. (1971) studied the unsonicated PC-water system by comparing a 220 MHz continuous wave spectrum with a 100 MHz pulse Fourier transform spectrum from which all T_2 times less than 250 μs were excluded.

Most of the $-\overset{+}{N}(CH_3)_3$ resonance and $-CH_3$ resonance signals but only some of the $-CH_2-$ resonance intensity observed in the 220 MHz spectrum appeared in the pulse spectrum. The distribution of T_2 times indicated that molecular motion is greatest at the ends of the molecule. The observation of a single T_1 time for all protons was taken to mean that relaxation occurs by spin diffusion through efficient spin-spin coupling to heat sinks effectively coupled to the lattice. Chan et al. (1971) judged that only the terminal methyl of the hydrocarbon chain or some $-CH_2-$ slightly further up the chain had sufficient mobility and an effective enough coupling to be the relaxation sink.

The identity of T_1 values for unsonicated PC was also observed by Daycock et al. (1971) who interpreted the phenomenon similarly, with spin diffusion taking place and the whole molecule relaxing by a single mechanism. However, their choice of relaxation sink was different. They judged that, below the transition temperature, the $-CH_3$ end of the hydrocarbon chain would not be sufficiently mobile. Above the transition temperature, perdeuterated PC exhibited the same behaviour as normal PC. Since the perdeuterated $-CH_3$ would not be satisfactory as a sink, they proposed the choline group.

The importance of spin diffusion as a relaxation mechanism of unsonicated PC in water was also emphasized by Charvolin and Rigny (1972) based on the small intensity

of the dipolar signal as compared with that of the free induction decay. The observation of slightly different T_1 times for the $-CH_3$ and $-CH_2-$ resonances precludes the role of the chain $-CH_3$ as a relaxation sink (Seiter et al. 1972).

Considerable controversy exists about the nature of the dramatically improved resolution of the NMR signal after sonication. T_1 values were found by Penkett et al. (1968) to be higher for the sonicated samples and so the authors mentioned the possibility of "some alteration of the intermolecular spacing... giving greater freedom of motion to the chains." However, they attributed the line narrowing mainly to the averaging out of magnetic anisotropies by rapid rotation and Brownian movement. In contrast, Sheard (1969) considered that the increase in Brownian motion is insufficient to account for the narrowing observed and therefore concluded that a change in structure must occur. Other workers (Finer et al. 1971, Finer 1972, Finer et al. 1972a, Chapman et al. 1972, Lee et al. 1972) remained convinced that the tumbling rate of sonicated vesicles is a significant factor that accounts for the narrower linewidths by averaging dipolar effects.

Using dipalmitoyl PC or egg PC sonicated in water, Lee et al. (1972) demonstrated separate T_1 relaxation times for the $-CH_3$, $-CH_2-$ and $-\overset{+}{N}(CH_3)_3$ signals. Since

the T_1 times are not equal, spin diffusion cannot be the principal relaxation mechanism here. ^{19}F NMR of monofluoro derivatives of stearic acid incorporated into PC vesicles (Birdsall et al. 1971) showed an approximately linear increase in disorder within the PC molecule from the glycerol moiety to the $-CH_3$ end. Using ^{13}C NMR, it was possible to observe progressively longer T_1 times (increasing molecular motion) on going from the glycerol to either the $-CH_3$ or the $-\overset{+}{N}(CH_3)_3$ ends of the molecule (Metcalfe et al. 1971). On the basis of these experiments on sonicated PC, Lee et al. (1972) refuted the interpretations of Chan et al. (1971), Daycock et al. (1971), and Charvolin and Rigny (1972) that spin diffusion is the principal mechanism for relaxation in unsonicated phosphatidyl choline bilayers.

Horwitz et al. (1972), using Fourier transform NMR of sonicated PC, obtained T_1 and T_2 relaxation times for the $-\overset{+}{N}(CH_3)_3$, $-CH_2COO$, $-CH_2-$, $-CH_2CH=CH$, $-CH=CH-$, and $-CH_3$ proton resonances. The T_1 of the main hydrocarbon chain $-CH_2-$ resonance appeared to have a single value, suggesting spin diffusion over short segments of the methylene chain as a possible relaxation mechanism, but the authors did not rule out the possibility of an undetected distribution of T_1 times. The T_2 values and, to some extent, the T_1 values indicated that a gradient of mobility exists along the hydrocarbon chain of the phospholipid molecule.

Similar conclusions had been drawn previously from ESR experiments. Fatty acids, spin labelled at different positions along the hydrocarbon chain, were either introduced directly into dipalmitoyl PC vesicles (Oldfield and Chapman 1971, Hubbell and McConnell 1971) or were used to synthesize spin labelled PC which was then incorporated into dipalmitoyl PC vesicles (Hubbell and McConnell 1971). From an examination of the ESR spectra order parameters, it was possible to show that the bilayer chain motion is most restricted towards the glycerol end and most fluid towards the $-CH_3$ end. However, Hubbell and McConnell (1971) admitted that spin label molecules "always represent some perturbation of the natural state of these systems." NMR techniques have the advantage of avoiding this difficulty.

The motion of the hydrocarbon chains can be described in terms of C-C trans-gauche isomerizations (Batchelor et al. 1972, Sheetz and Chan 1972, Horwitz et al. 1972, Horwitz 1972). These authors assumed that vesicle tumbling is not a significant motion contributing to nuclear relaxation and that the greater curvature of the sonicated vesicles, as compared with the multilayered aggregates, loosens the packing of the chains, facilitating trans-gauche transitions especially near the $-CH_3$ end (Sheetz and Chan 1972, Horwitz et al. 1972, Chan et al. 1973). These would either be β-coupled transitions (Horwitz et al. 1973) or some other form of kink (Seiter

and Chan 1973) which would not greatly disturb the packing. These transitions would provide the primary relaxation mechanism for T_2 while small rotations about C-C bonds would contribute most to T_1.

Theoretical treatments of both vesicle tumbling and molecular rearrangements after sonication have been published. Horwitz et al. (1973a) predicted that if tumbling were important, then, contrary to experimental observations, single T_2 times would be observed for all protons and linewidths would be sensitive to viscosity.

Chan et al. (1972) applied the treatment of Woessner (1962) to unsonicated PC. This is a treatment of magnetic dipole-dipole relaxation of spins attached to groups undergoing anisotropic motion. The main feature is a separation of the correlation time into a component related to the motion of a rotor axis, $\tau_\perp$, and a component related to the motion of the protons about the axis, $\tau_\parallel$. T_1 is determined by $\tau_\parallel$, the faster process. The effect of the orientational distribution of these axes relative to the applied magnetic field was considered to be the cause of a distribution of T_2 times. The variation of T_2 due to axis orientation was verified experimentally.

This analysis was expanded by Seiter and Chan (1973) to cover theoretical linewidths. The stochastic Anderson theory (1954), as applied to the problem of motional averaging of dipolar broadening by Saunders and Johnson (1968), was modified by restricting the possible

orientation of the internuclear vector in accordance with a physical picture of hydrated phospholipids. Above the transition temperature, the experimentally determined behaviour of the $-CH_2-$ T_2 time and free induction decay was explained in terms of reorientation of the protons about an axis which itself has considerable motion. Fluctuations of intermolecular dipole fields due to diffusion were also considered for the $-CH_2-$, using the Kruger (1969) approach. The narrowness of the $-CH_3$ and $-\overset{+}{N}(CH_3)_3$ linewidths was interpreted in terms of intramolecular dipole fields from the neighbouring $-CH_2-$ or $-NCH_3$ groups respectively combined with a fairly restricted motion of the choline and chain methyl groups.

The Anderson theory was also used to demonstrate the insignificance of vesicle tumbling on relaxation rates and the need to postulate a much greater molecular motion in sonicated PC than in the multilayers.

Finer (1974) asserted that the treatment of Woessner (1962) is not applicable when a motion is so slow that its correlation time is of the order of T_2. In this case, T_2 is made up of a slow overall aggregate motion and a more rapid segmental molecular motion. The Gutowsky and Pake second moment method (1950) for solids with nuclei experiencing "specialized motion", in this case anisotropic motion, was modified by introducing a correlation time for tumbling. The second moment becomes a sum of two terms, one reduced by tumbling, the other dependent on both tumbling

and internal motion. The use of the observed linewidth in these calculations rather than the rigid lattice linewidth was advocated by reference to theoretical and experimental considerations. It was then possible to explain the reduction of linewidth caused by sonication, the insensitivity of linewidth to viscosity, and the importance of vesicle size for the linewidth. The theory also accomodates different relaxation rates for different protons.

Lichtenberg et al. (1975) have stated that Finer's (1974) approach is not suitable for phospholipid vesicles. The Gutowsky-Pake theory was considered appropriate only for lines inhomogeneously broadened by static dipolar interactions whereas the phospholipid spectrum lines were considered to be only partly inhomogeneous. Lichtenberg et al. (1975) also regarded the rigid lattice linewidth as the valid parameter for calculations of correlation times. They therefore reasserted that the greater surface curvature of the small vesicles causes variations in the molecular packing.

Metcalfe et al. (1973) and Lee et al. (1973) found that they could not explain proton relaxation simply from intramolecular dipole-dipole interactions but had also to include intermolecular interactions because otherwise the ^{1}H NMR did not indicate the same quantitative gradient of chain mobility as did ^{13}C NMR. They first estimated theoretical intramolecular relaxation rates expected for

isotropic systems. In this case, the proton T_1 should be greater than the ^{13}C T_1. Since experimentally, the reverse is true and since the ^{13}C relaxation processes are known to be less affected by intermolecular effects, they concluded that proton NMR relaxation rates do have a contribution from intermolecular effects. However, it is generally accepted that the motion of lecithin molecules in bilayers is anisotropic and therefore, the theoretical basis for their conclusion was not satisfactory. On the other hand, they found that mixtures of perdeuterated dipalmitoyl PC with non-deuterated PC exhibited increased T_1 times while the linewidth decreased. Because deuterium has a smaller magnetic dipole moment, it is less effective in relaxing nuclei. That it should cause increased T_1 times in the normal PC is a sign that intermolecular effects are present. Using this interpretation, calculated self-diffusion coefficients for the lipids in sonicated vesicles were very similar to the lateral diffusion rates for spin-labelled lecithins in unsonicated PC bilayers (Devaux and McConnell 1972).

The picture is further complicated by the possibility of different relaxation mechanisms operating in different parts of the same molecule. Intermolecular effects could be most important at the hydrocarbon methyl, oscillation of the fatty acid chains perpendicular to the plane of the bilayer could operate for $-CH_2-$'s higher up the chain, while vesicle tumbling could affect the chain region nearest the glycerol group (Metcalfe et al. 1973).

Blood Coagulation: Molecular Characteristics

In the earliest theories of blood coagulation, (Morawitz 1905), it was recognized that tissue extracts are required for the formation of fibrin clots. Further research indicated that the phospholipid component of tissue extracts is essential (Mills 1921). The interaction of lipids with the various clotting factors has been depicted by Hemker et al. (1970), based on the scheme proposed by Barton (1967), as shown below. The numbering system is that of the International Committee on the Nomenclature of Blood Clotting Factors. A list of synonyms is presented in Table I. The subscript 'a' refers to activated enzymatic forms of the factors, except in the case of fibrin, where the subscripts 'a' and 'b' refer to a lower molecular weight form and the ε-lysine-γ-glutamyl crosslinked form respectively.

XII	---------▷	XIIa
XIIa + XI	---------▷	complex C
IX	complex C ▷	IXa
IXa +VIII +PL+ Ca^{++}	---------▷	complex B
X	complex B ▷	Xa
Xa + V + PL + Ca^{++}	---------▷	complex A
II	complex A ▷	IIa
I	---IIa---▷	Ia
Ia	--XIIIa--▷	Ib

where PL is phospholipid

TABLE I

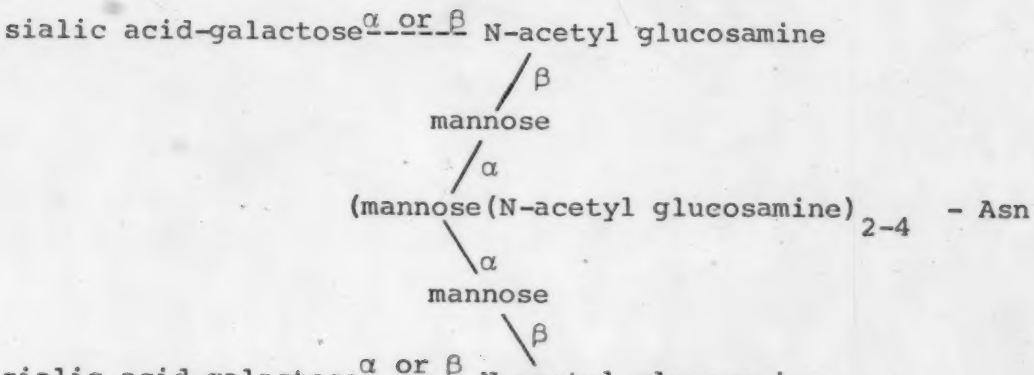
BLOOD CLOTTING FACTOR NOMENCLATURE

Roman Numeral	Preferred Name	Synonyms
I	Fibrinogen	
II	Prothrombin	
IV	Calcium ions	
V	Proaccelerin	Accelerator globulin, Labile factor
VII	Proconvertin	SPCA, Stable factor, Autoprothrombin I
VIII	Antihaemophilic factor	Antihaemophilic globulin, Platelet cofactor I
IX	Plasma thrombo-plastin component	Christmas factor, Autoprothrombin II
X	Stuart-Prower factor	Autoprothrombin III, Prothrombokinase
XI	Plasma thrombo-plastin antecedent	Platelet factor II, Antihaemophilic factor G
XII	Hageman factor	Glass factor, contact factor, surface factor
XIII	Fibrin stabilizing factor	Fibrinase, Laki-Lorand factor

(From Wright 1962, Josso et al. 1964)

This thesis is concerned with the interaction of lipids with prothrombin. Prothrombin is a plasma glycoprotein. At the commencement of this work, the method of choice for its isolation from bovine plasma was based on $BaSO_4$ adsorption, sodium citrate elution, DEAE-cellulose chromatography and Sephadex gel chromatography, according to the method of Cox and Hanahan (1970) as modified by Bull et al. (1972). Other methods have been described by Seegers (1952), Lewis and Ware (1953), Milstone (1955), Goldstein et al. (1959), Lanchantin et al. (1963), Moore et al. (1965), Magnusson (1965), Tishkoff et al. (1968), Ingwall and Scheraga (1969) and Mann et al. (1971).

The prothrombin molecule consists of a single peptide chain of molecular weight between 68,000 and 74,000 (Lamy and Waugh 1958, Harmison et al. 1961, Tishkoff et al. 1968, Cox and Hanahan 1970). The carbohydrate content is 10-12% of the total weight (Magnusson 1965, Lanchantin et al. 1968, Cox and Hanahan 1970). According to Magnusson (1965), sialic acid-galactose-mannose-glucosamine-protein is the predominant type of linkage although small amounts of galactosamine were also found. Nelsestuen and Suttie (1972) detected both α and β galactose residues and proposed that two sequences are present: one with α galactosidic bonds and one with β galactosidic bonds,



They stated that there are four such carbohydrate chains in prothrombin, each of which is linked to an asparagine residue.

The amino acid compositions of rat, bovine, and human prothrombins have been determined (Li and Olson 1967, Magnusson 1964, Lanchantin et al. 1968). All are similar. There are many glutamic and aspartic acid residues. Four disulphide bonds are easily accessible and four are revealed only on treatment with urea (Carter 1959). There are no free sulphydryl groups (Carter 1954, Carter and Warner 1954, Carter and Warner 1956, Laki et al. 1954). The N-terminal residue is alanine (Magnusson 1958) and the C-terminal serine (Thomas and Seegers 1960). Partial amino acid sequences have been reported (Magnusson 1970, 1970a, Hartley 1970, Magnusson et al. 1973, 1974).

Many authors have investigated the Ca^{++} binding properties of prothrombin. Barton (1971) found 4 higher affinity binding sites (log K_a 4) and approximately twelve lower affinity sites (log K_a 2.54). Nelsestuen

and Suttie (1972a) reported that at 1 mM Ca^{++}, four moles of Ca^{++} were bound cooperatively per 70,000g of protein. Stenflo and Ganrot (1973) found cooperative binding of 3 higher affinity sites and 7-9 weaker binding sites. Benson et al. (1973) reported the existence of ten sites (log K_a 3.20) and according to Bajaj et al. (1974), prothrombin has 10 or 11 Ca^{++} binding sites, 6 of which have log K_a 3.5 and 4 or 5 with log K_a 2.7.

Activated Factor X (Xa) is a serine protease which hydrolyzes prothrombin to thrombin directly (Barton et al. 1967) but only at a very slow rate, inadequate for hemostasis (Milstone 1964). When Factor V, a protein cofactor possessing no demonstrable enzymatic activity (Hemker et al. 1967, Barton et al. 1967, Jobin and Esnouf 1967) is added to Factor II and Factor Xa in the presence of phospholipid and Ca^{++}, there is a large increase in the rate of thrombin formation (Milstone 1964, Papahadjopoulos and Hanahan 1964, Cole et al. 1965, Jobin and Esnouf 1967).

All phospholipids are not equally effective procoagulants. Earlier attempts to identify the most active phospholipid classes produced rather variable results and conclusions due partly to the use of different blood clotting assays in different laboratories and partly to the use of preparations of lipids of dubious purity. These investigations have been summarized by

Marcus (1966). In the main, phosphatidyl serine (PS) or mixtures of PS with PC appear to be most active fractions. However, this does not appear to be true for the reactivation of delipidated tissue factor (Nemerson 1968) and high concentrations of PS have been reported to be inhibitory (Silver et al. 1957, Mustard et al. 1962).

In 1961, Bangham demonstrated a relationship between the surface charge density of phospholipids and their " thromboplastic " (procoagulant) activity. Similar observations have subsequently been made by many other workers (Papahadjopoulos et al. 1962, Silver et al. 1963, Daemen et al. 1965). However, Barton et al. (1970) have shown that, at identical electrophoretic mobilities, mixtures of phosphatidic acid (PA) and PC are in fact much less active than mixtures of PS and PC. Therefore, factors other than charge density alone are important. For example, the ability to chelate Ca^{++} and the geometrical structures of Ca^{++}-lipid complexes may be important. The structure of the hydrocarbon chains of the phospholipid molecules, dependent on the fatty acid composition and the temperature, are also significant parameters. Fully saturated phospholipids were found to have much lower clot promoting activity than mixed or unsaturated phospholipids (Rouser and Schloredt 1958, Rouser et al. 1958, Wallach et al. 1959). This is due to the fact that at physiological temperatures, the former are in a gel

state while the latter are in the liquid crystalline state (Sterzing and Barton 1973). When cholesterol, which increases the chain motion of phospholipids in the gel state, was added to fully saturated phospholipids, the clotting activity was considerably enhanced (Sterzing and Barton 1973).

A complex of phospholipid, Ca^{++}, and Factors V and Xa was sedimented by ultracentrifugation (Cross 1962, Cole et al. 1965, Jobin and Esnouf 1967) and could be separated from its individual protein components by gel filtration (Papahadjopoulos and Hanahan 1964). Factor V alone bound to phospholipid mixtures without Ca^{++} or at low concentrations (0.005 M Ca^{++}) (Papahadjopoulos and Hanahan 1964, Cole et al. 1965, Jobin and Esnouf 1967). In contrast, both Factor Xa (Papahadjopoulos and Hanahan 1964, Jobin and Esnouf 1967, Barton et al. 1967) and prothrombin (Hanahan et al. 1969, Barton and Hanahan 1969) require Ca^{++} for binding to phospholipids. Thrombin does not bind to phospholipid either in the presence or absence of Ca^{++} (Barton and Hanahan 1969).

More complete binding studies were undertaken by Bull et al. (1972) and Bull (1973) using gel filtration at pH 9 in the presence of Ca^{++}. Increased binding of prothrombin was correlated with increased prothrombin activity in clotting tests as the percentage of PS in a mixture of PS and PC was raised from 0 to 30%. Above 30%, the same amount of prothrombin was bound but

coagulant activity decreased. Pure PC or mixtures of PC and PE bound very little prothrombin and were inactive. Pure PS, pure PA, mixtures of PS and PA, or PC and PA at high PA concentrations strongly bound prothrombin but the activity of the complexes was much lower. Bound prothrombin could be more easily removed from PS:PC than from PS:PA by EDTA.

It was found that treatment of prothrombin with Ca^{++} altered its conformation as inferred from ultracentrifugal sedimentation analysis, ultraviolet spectra (Bull 1973) and the aromatic CD spectrum (Björk and Stenflo 1973). It was suggested that prothrombin binds to phospholipids through Ca^{++} chelation (Rouser and Schloredt 1958, Barton and Hanahan 1969). A model of prothrombin activation was proposed by Barton and Hanahan (1969) and Barton (1970). The key features were

a) binding of prothrombin to the lipid surface

b) conversion of prothrombin to thrombin as a result of the action of Factors Xa and V at the lipid surface

c) release of thrombin into the bulk aqueous phase.

This mechanism was able to account for the results of kinetic analyses revealing no product inhibition of the prothrombin to thrombin conversion.

Although several authors had detected intermediates in the conversion of prothrombin to thrombin (Seegers and Marciniak 1965, Lanchantin et al. 1965,1968a, Aronson and Ménaché 1966, Ganrot and Nilehn 1969), it was not until recently that a clear understanding of the

possible activation pathways emerged. Using gel electrophoresis, Mann et al. (1971), Stenn and Blout (1972), Heldebrant and Mann (1973), Heldebrant et al. (1973), Owen et al. (1974), and Esmon et al. (1974) were able to follow the appearance of fragments during activation. The composite scheme is shown in Figure 2.

Gitel et al. (1973) isolated prothrombin, intermediate 1 and fragment 1. They examined the binding of each to equimolar mixtures of synthetic dioleoyl phosphatidyl glycerol (PG) and PC in the presence of Ca^{++}. It was found that only fragment 1 and prothrombin itself would bind to this lipid mixture. Fragment 1 also bound to dioleoyl PE:PG and cephalin, an isolated brain lipid fraction, predominantly PS and PE. This polypeptide has a large number of acidic as well as a large number of apolar residues. Thus, it was shown that prothrombin contains a specific phospholipid binding area which is not a part of the thrombin molecule (Gitel et al. 1973). Benson et al. (1973) carried the analysis of prothrombin, fragment 1, and intermediate 1 further to study carbohydrate content, N-terminal amino acid residues and Ca^{++} binding properties. Fragment 1 contains most of the sialic acid and neutral hexoses and is derived from the N-terminal region of prothrombin. It exhibited 12-15 Ca^{++} binding sites (log K_a 3.17) which is at least as many as in prothrombin. Intermediate 1 has no such sites. Slightly different results were obtained by Bajaj et al. (1974). According

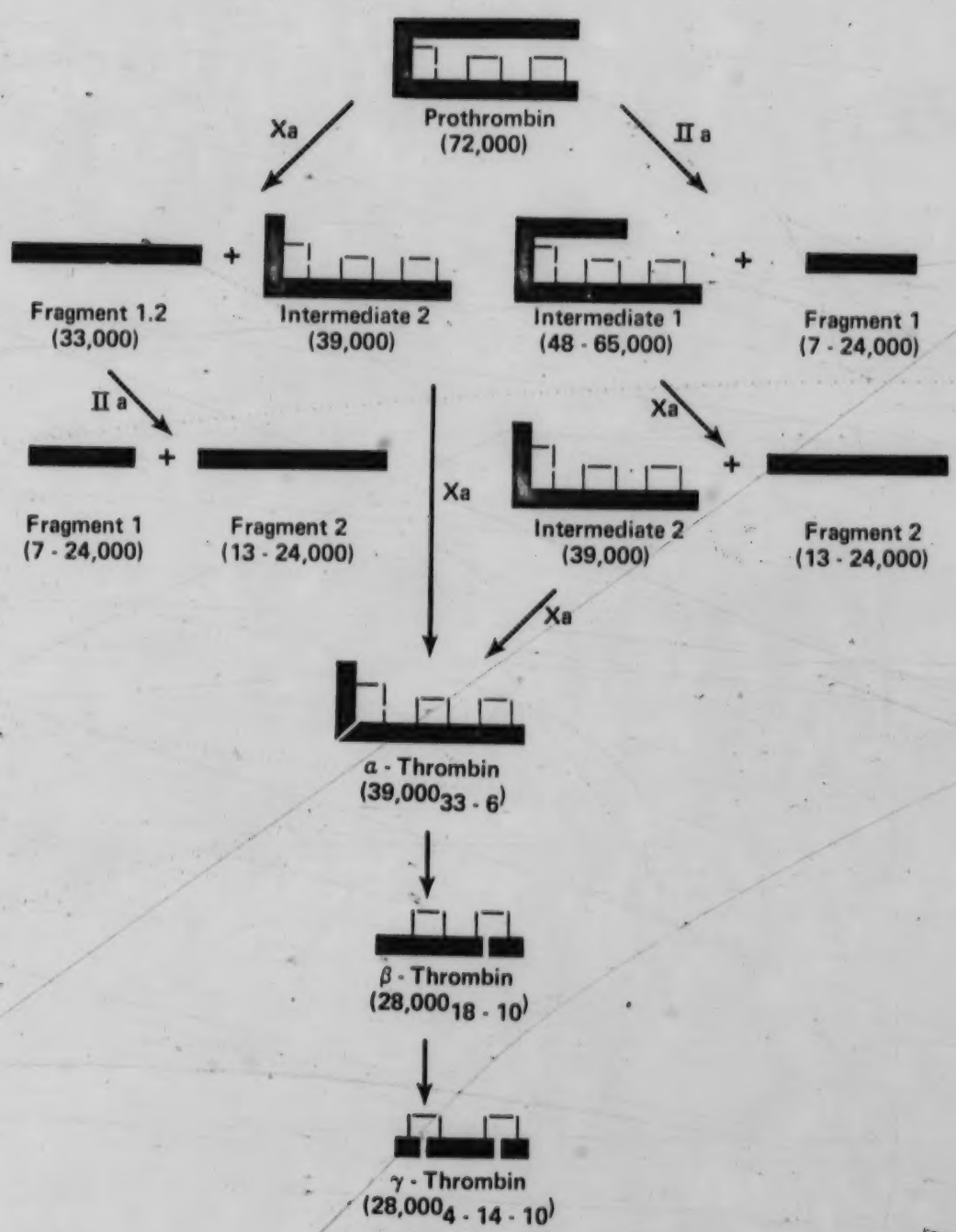


Figure 2. Diagrammatic representation of the process of prothrombin activation. Disulphide bonds are indicated by — — —. Molecular weights are given in parentheses with subscripts referring to the molecular weight of the constituent polypeptide chain in thousands.

to them, the six stronger binding sites of prothrombin were found on fragment 1 while the 4 to 5 weaker sites were on the fragment 2 section of intermediate 1.

Using SDS-acrylamide gel electrophoresis and gel filtration chromatography, Jackson and associates have examined the interactions between the various prothrombin activation products and Factors V, Xa, and phospholipid (Jackson et al. 1974, Esmon and Jackson 1974, 1974a, Esmon et al. 1974a, 1974b). The results are summarized in Table II. In addition, kinetic analyses based on thrombin generation were performed. Based on the results, a plausible model was proposed, as follows (Jackson et al. 1974, Esmon et al. 1974b).

Factors Xa and II bind to phospholipid through Ca^{++} chelation while Factor V does not require Ca^{++} for binding. On the lipid surface, Factor Xa hydrolyzes prothrombin to intermediate 2 and fragment 1.2. Fragment 1.2 remains bound to the phospholipid through its fragment 1 region. Its fragment 2 region binds non-covalently both to intermediate 2 and to Factor V. Factor V is also bound to Factor Xa and thus facilitates the second proteolysis of intermediate 2 into the two-chain thrombin. Presumably thrombin cannot bind to fragment 1.2 and is released into the bulk aqueous phase where it splits prothrombin into intermediate 1 and fragment 1. Since intermediate 1 cannot bind to phospholipid, its hydrolysis by Factor Xa in the bulk phase is slow.

TABLE II

COMPLEX FORMATION DURING PROTHROMBIN ACTIVATION

Species present	Ca^{++} dependence	Complex formation	Reference
II, PL, Ca^{++}	yes	yes	Jackson et al. 1974
Fragment 1, PL, Ca^{++}	yes	yes	Gitel et al. 1973
Intermediate 1, PL, Ca^{++}	---	no	Gitel et al. 1973
IIa, PL, Ca^{++}	---	no	Jackson et al. 1974
II, V, Ca^{++}	---	no	Esmon et al. 1973
II, Va*, Ca^{++}	---	yes	Esmon et al. 1973
Fragment 1, Va, Ca^{++}	---	no	Esmon and Jackson 1974a
Intermediate 1, Va, Ca^{++}	---	yes	Esmon and Jackson 1974a
Fragment 2, Va, Ca^{++}	---	yes	Esmon and Jackson 1974a
Fragment 1.2, Va, Ca^{++}	---	yes	Esmon and Jackson 1974a
Fragment 2, Intermediate 2	---	yes	Esmon and Jackson 1974a
Fragment 1.2, Intermediate 2	---	yes	Esmon et al. 1974a
V, Xa, Ca^{++}	yes	yes	Papahadjopoulos and Hanahan 1964
PL, Xa, Ca^{++}	yes	yes	Papahadjopoulos and Hanahan 1964
V, PL	no	yes	Papahadjopoulos and Hanahan 1964
Va, PL	no	yes	Papahadjopoulos and Hanahan 1964

* Va is a modified Factor V. Authors have often not distinguished between these forms.

Role of Vitamin K

The presence of Ca^{++} binding sites in the prothrombin molecule is apparently dependent on the action of vitamin K during prothrombin biosynthesis. If a vitamin K deficiency exists, or if vitamin K antagonists, such as dicoumarol or warfarin are fed to animals, abnormal prothrombins are produced (Malhotra 1972, 1972a, Stenflo 1972). The normal and abnormal prothrombins have similar molecular weights, amino acid compositions, amino- and carboxy-terminal amino acids, antigenic determinants and electrophoretic mobilities on disc gels at pH 2.7, 7.0, 8.9 and 9.5 (Malhotra and Carter 1971, Nelsestuen and Suttie 1972b, 1972c, Stenflo and Ganrot 1972). Their peptide maps and carbohydrate compositions are also very similar (Nelsestuen and Suttie 1972b, Johnson et al. 1972). According to Hemker et al. (1968), the abnormal prothrombin competitively inhibits the conversion of prothrombin to thrombin.

Although differences in structure between normal and abnormal prothrombin must be slight, certain of their physical and biological properties vary markedly. The abnormal prothrombin binds only one g atom of Ca^{++} per mole of protein with an apparent log K_a of 4.30 (Nelsestuen and Suttie 1972a, Stenflo and Ganrot 1973). It is poorly adsorbed by barium citrate, in sharp contrast to prothrombin. According to Johnson et al. (1972a), it cannot be activated to thrombin under physiological conditions (lipid-dependent

mechanism). However, Malhotra (1972, 1972a) found slow partial activation in a system of Factors V, VII, X and Ca^{++}. Trypsin treatment generated thrombin activity (Nelsestuen and Suttie 1972b) as did storage in 25% sodium citrate or in protamine sulphate solutions (Malhotra and Carter 1972). These are catalytic mechanisms that operate independently of phospholipids. This suggested that the difference between normal and abnormal prothrombin resides in the ability of the fragment 1 component of prothrombin to bind lipid through Ca^{++} bridges.

Olson's group (Li et al. 1970) observed an increased precipitation of labelled prothrombin-anti-prothrombin complexes in the perfusates of isolated rat livers from warfarin-treated rats after vitamin K addition. No prothrombin antigens could be found in liver microsomes of vitamin K-deficient rats but when vitamin K was added, greater than normal amounts of prothrombin could be detected (Johnston and Olson 1972). It was thought at that time that vitamin K affected the initiation of prothrombin synthesis by ribosomes.

In support of this, cycloheximide, a translation inhibitor, reduced the increase in active prothrombin production normally observed after vitamin K administration (Kipfer et al. 1972). Physico-chemical and immunological investigation led Morrissey et al. (1972) to state that the properties of abnormal prothrombin found by other

groups was so different from that of normal prothrombin that in fact it was not related to prothrombin at all.

Earlier, Hemker et al. (1968) had hypothesized that the vitamin K-dependent step was a post-translational addition of carbohydrate. Pereira and Couri (1970), on the basis of labelling experiments with glucosamine and leucine, reported that dicoumarol inhibited glucosaminyl transferase. Cycloheximide was found by them and others (Hill et al. 1968, Suttie 1970, Shah and Suttie 1971, 1972) to have very little inhibitory effect on the increased prothrombin activity when vitamin K was added to dicoumarol-treated rats. This implies that the action of vitamin K is at a post-ribosomal stage (Shah and Suttie 1971, 1972). Johnson et al. (1971a, 1972a) found that vitamin K did not influence amino acid incorporation into prothrombin. They then concluded that vitamin K was not involved in protein biosynthesis. Other labelling experiments supported the view that the action of vitamin K was at a stage of carbohydrate attachment (Johnson et al. 1971a). It was also reported that abnormal prothrombin (M.W. 68,000) had a much lower molecular weight than normal prothrombin (M.W. 91,000) as measured by SDS-acrylamide gel electrophoresis (Johnson et al. 1972).

Nelsestuen and Suttie (1971) removed most of the sialic acid and 40% of the hexosamines from normal prothrombin by treatment with glycosidase enzymes. The modified prothrombin retained 70% of the original

specific activity and was adsorbed on barium citrate. They concluded that vitamin K was not implicated in glycosylation. Instead, they suggested that vitamin K might be involved in the incorporation of an unknown prosthetic group into the polypeptide chain. The possibility that vitamin K causes a change in tertiary structure or in the disulphide bonds was ruled out because reduction and subsequent re-oxidation caused no change in the high affinity of normal prothrombin for Ca^{++} or in the low affinity of abnormal prothrombin (Nelsestuen and Suttie 1972a). Stenflo (1972) did find that separation of normal and abnormal prothrombin occurred in polyacrylamide gel electrophoresis at pH 8.9 in 8 M urea if the proteins were not reduced and alkylated.

Localization of the vitamin K-dependent Ca^{++} binding sites has been partially achieved by analysis of prothrombin activation products. Stenflo (1973) used thrombin to digest normal and abnormal prothrombins. In each case, two large polypeptides, intermediate 1 and fragment 1, were obtained. Only fragment 1 from normal prothrombin bound Ca^{++}. Amino acid and carbohydrate analyses of the fragments 1 from normal and abnormal prothrombins were identical, as were peptide maps of reduced and amino-ethylated tryptic digests. However, thermolysin digestion of reduced and non-reduced fragments 1 gave clearly distinguishable peptide maps. Immunoelectrophoresis in the presence of Ca^{++} also showed differences in the

antigenic properties of normal prothrombin fragment 1 and abnormal prothrombin fragment 1 (Stenflo 1973).

Cyanogen bromide degradation of fragment 1 followed by tryptic digestion and peptide mapping yielded two small peptides from normal prothrombin which were not obtained from abnormal prothrombin (Stenflo 1974). Both peptides contained Ca^{++} binding sites. The smaller peptide, a heptapeptide, had an electrophoretic mobility which would only be consistent with its amino acid composition if a prosthetic group exhibiting 3 negative charges was attached to it. In contrast, the corresponding peptide from abnormal prothrombin had an electrophoretic mobility consistent with its amino acid composition. A second and larger peptide (22 or 23 residues) from normal prothrombin contained two arginyl bonds that were not hydrolyzed by trypsin. One of these arginyl residues was known to be flanked by glutamyl residues on both sides (Heldebrant et al. 1973). In abnormal prothrombin, these arginyl bonds are hydrolyzed by trypsin (Stenflo 1974). NMR and mass spectroscopy were then used to compare the isolated smaller peptide from normal prothrombin with a synthetic peptide of the same amino acid sequence. It was deduced from the results that the two glutamyl residues present were both substituted at the γ-position with an additional COOH group (Stenflo et al. 1974).

The larger peptide was also isolated directly from a tryptic digest of normal prothrombin by barium

citrate adsorption (Nelsestuen and Suttie 1973). It was found to bind Ca^{++}. No corresponding peptide could be adsorbed onto barium citrate after trypsin treatment of abnormal prothrombin. Based on the resistance of a protease-derived octapeptide to further proteolysis and on a disparity between dry weight and amino acid composition, Nelsestuen and Suttie (1973) also deduced that a prosthetic group was involved. Mass spectroscopy of a peptide isolated from a pronase digest of the reduced and carboxyamidomethylated larger peptide and deuterium exchange revealed an extra carboxyl group on the γ-carbon of the glutamyl residue (Nelsestuen et al. 1974).

The number of modified glutamyl residues in this larger peptide was determined by titration of the carboxyl groups and coupling of these to glycine ethyl ester (Howard and Nelsestuen 1974). A minimum of seven extra carboxyl groups were found. The authors suggested that probably all of the eight glutamyl residues are carboxylated.

The primary structure of the first forty two amino acid residues from the N-terminal was described by Magnusson et al. (1974). Extra carboxyl groups were found on all ten glutamyl residues, confirming previous results. Further research is required to correlate these modified residues with the Ca^{++} binding sites, and especially to differentiate between the strong and weak binding sites.

In vivo incorporation of $^{14}C\ CO_2$ into glutamyl residues of prothrombin was higher when vitamin K was

administered to warfarin-treated rats than when it was given to normal rats (Girardot et al. 1974). This provides an explanation for the increased incorporation of the ^{14}C label from ^{14}C glucosamine into prothrombin after vitamin K treatment (Pereira and Couri 1970, Johnson et al. 1971a) while the carbohydrate composition of prothrombin remained unchanged (Nelsestuen and Suttie 1972b).

The aim of the research

The aim of the research described in this thesis was to elucidate the nature of the interactions involved in the formation of prothrombin-Ca^{++}-phospholipid complexes. Four major types of interaction could be implicated (Salem 1962, Tanford 1973):

1) Formation of mixed calcium chelates with phospholipid and protein ligands appeared to be most important, in view of the dissociation of the complex by EDTA treatment (Bull 1973)

2) Electrostatic interactions between lipid and protein, involving primarily negatively-charged groups of the lipids and the positively-charged groups of the protein

3) A hydrophobic effect sequestering the hydrocarbon chains of the phospholipid with the apolar residues of the protein

4) London-van der Waals and hydrogen bonding

It was hoped that NMR could provide some information in order to distinguish the relative importance of at least the first three types of interaction. Lipid-protein electrostatic interactions would be expected to broaden the $-\overset{+}{N}(CH_3)_3$ line. Hydrophobic interactions

between the protein and the lipid should lead to broadening of the $-CH_2-$ resonance line.

In addition, it was of interest to investigate the effect of Ca^{++} on the phospholipid itself. Studies had been performed on monolayers (Bangham and Papahadjopoulos 1966, Papahadjopoulos 1968) showing a "condensation" of PS following addition of Ca^{++}. Interactions of this kind should also lead to broadening of the $-CH_2-$ resonance line. Calcium ion binding to the polar head groups of PS might be expected to affect the $-\overset{+}{N}(CH_3)_3$ resonance of PC molecules intercalated between PS molecules.

The objectives were to clarify this facet of blood coagulation and at the same time gain an insight into the more general problem of lipid-protein interactions in membranes.

CHAPTER II

MATERIALS AND METHODS

A. Materials

D_2O (99.77% pure) was obtained from Columbia Organic Chemicals (Columbia, S.C., U.S.A.). NaOD used to adjust pH was prepared by dissolving pellets of NaOH in D_2O. 2 M $CaCl_2$ in D_2O was prepared by dissolving either 2.94 g of $CaCl_2 \cdot 2H_2O$ or 2.22 g $CaCl_2$ in 10 ml of D_2O. Silica gel G (E. Merck) was obtained from Brinkmann Instruments (Toronto). Cytochrome c, Type III from horse heart, lot 87B-7131 (97.5% pure when corrected for 7.2% H_2O) or lot 92C-1800 (100%), was obtained from Sigma (St. Louis, Mo., U.S.A.). Standard normal plasma came from DADE (Miami, Fla., U.S.A.). Russell viper venom (Burroughs-Wellcome 'Stypven') was obtained from Warner-Chillcott (Scarborough, Ont.)

Cephalin or brain lipid extract was prepared according to Bell and Alton (1954) at a concentration of 116 μg P/ml and diluted to 2 μg/ml for use in assays. Prothrombin was prepared according to Cox and Hanahan (1970) with the citrate elution from DEAE replaced by the step-wise (0.05M, 0.075M, 0.1M) and gradient (0.1M pH 7.2 to 0.175M pH 7.45) phosphate elution procedure of Seegers and Landaburu (1960) that was adopted by Bull (1973). After the final Sephadex chromatography, the prothrombin was stored in 0.1M NaCl, 10^{-4}M DFP at pH 6.8 in small tubes,

in the dark and at -20°C. PC was purified from hen egg yolk by silicic acid chromatography according to Lea et al. (1955) as modified by Hanahan et al. (1957). PS was prepared from frozen beef brains essentially by the method of Sanders (1967). Both PC and PS were stored as solutions in chloroform at -20°C.

B. Methods

1. Phosphorus determinations: Phospholipid phosphorus was determined according to King (1932). Calculations assumed a phospholipid molecular weight of 775.

2. Protein determinations: for cytochrome c, the dry quantity was weighed. For prothrombin, concentrations were determined by absorbance of solutions at 280 nm. ($E^{1\%}_{1\ cm}$ = 14.0 according to Cox and Hanahan 1970).

3. Phospholipid sample preparations: phospholipid in chloroform was evaporated under a N_2 stream. Care was taken, as the volume became small, not to trap chloroform under the skin of lipid which formed. The tubes were covered and placed in a vacuum chamber for 20 to 24 hours in order to remove any remaining traces of chloroform. D_2O was added and swelling was allowed to occur for another 20 to 24 hours. The samples were then frozen and lyophilized overnight. The swelling-lyophilizing sequence was repeated, the samples swollen for a third time for a 24 hour period before sonication.

When PS:PC mixtures were required, chloroform solutions of the lipids were mixed prior to the above steps.

4. Sonication: The dispersions were sonicated using a Biosonik sonic oscillator (Bronwill Scientific, Rochester, N.Y., U.S.A.) at a power level setting of 40 to 50. Alternatively, for a few samples, a Biosonik III at levels of 40 to 50 was used. Samples were under a nitrogen stream and in an ice-water bath. Sonication time was routinely 25 minutes, in periods of 5 minutes with 5 minute intervals between operation. pH was adjusted during these intervals, usually to between pH 7.1 and 7.7.

For phospholipid-Ca^{++} samples, the $CaCl_2$ was added to previously sonicated phospholipid and resonicated for another 25 minutes. Final Ca^{++} concentration was 0.01M.

5. Nuclear Magnetic Resonance: Spectra were obtained on a 100.0 MHz Varian HA-100-15 spectrometer either with continuous wave or pulse techniques. Routine operating temperature was 29 to 31°C. Assignments were made according to Chapman and Morrison (1966).

6. Fatty acid analysis: Phospholipids were digested in sulphuric acid and the fatty acids esterified with methanol according to Gander et al. (1962). The methyl esters were then run on a Hewlett-Packard 5700A gas chromatograph equipped with a diethylene glycol succinate column. The analysis was run either isothermally or with a temperature program consisting of 16 minutes at 200°C then a rise of 2°/min to 240°C. Peaks were identified by reference to standards from Nu-Check Prep (Elysian, Minn., U.S.A.). The equivalent chain length (ECL) method of Miwa et al.

(1960) and Woodford and van Gent (1960) was used to identify peaks for which no standard was available. Values of ECL were taken from Jamieson (1970).

7. Thin-layer chromatography: Chromatographic separation of lipids on thin-layer plates coated with silica gel G was achieved with $CHCl_3$: CH_3OH: 6N NH_4OH (65/35/5 v/v/v), $CHCl_3$: CH_3OH: 6N CH_3COOH (65/35/5 v/v/v), or petroleum ether: diethyl ether: CH_3COOH (90/10/trace).

8. Amino acid analysis: amino acid analysis of prothrombin was carried out with a Durrum D-500 amino acid analyzer. Samples were hydrolyzed in 6N HCl, 0.1% phenol at 110°C for 24 (duplicates), 48 and 78 hours (single samples) in sealed, evacuated tubes. Peak areas were computer analyzed. Values taken were those at 78 hours except for serine and threonine which were obtained by extrapolation to zero time, and half-cystines calculated from the 24 hour data.

9. Electrophoresis: The prothrombin sample was treated with 1% β-mercaptoethanol and 1% SDS then subjected to SDS-acrylamide disc gel electrophoresis on 10% gels in 0.1 M phosphate buffer, pH 7.2 with 0.1% SDS, 0.27% methylenebisacrylamide according to the method of Weber and Osborn (1969).

10. Coagulation assays: Prothrombin activity was assayed by the Russell viper venom (RVV) - cephalin assay of Hjört et al. (1955), using Factor II and VII-deficient plasma. The method was calibrated by reference to the clotting

times obtained with dilutions of standard human plasma. These are plotted on a log-log graph of the type shown in figure 3.

11. Ultracentrifuge experiments: Measurements were made on a Beckman Model E ultracentrifuge operating at 20°C and equipped with Schlieren optics. Sedimentation coefficients were obtained by a computer least-squares analysis of the data.

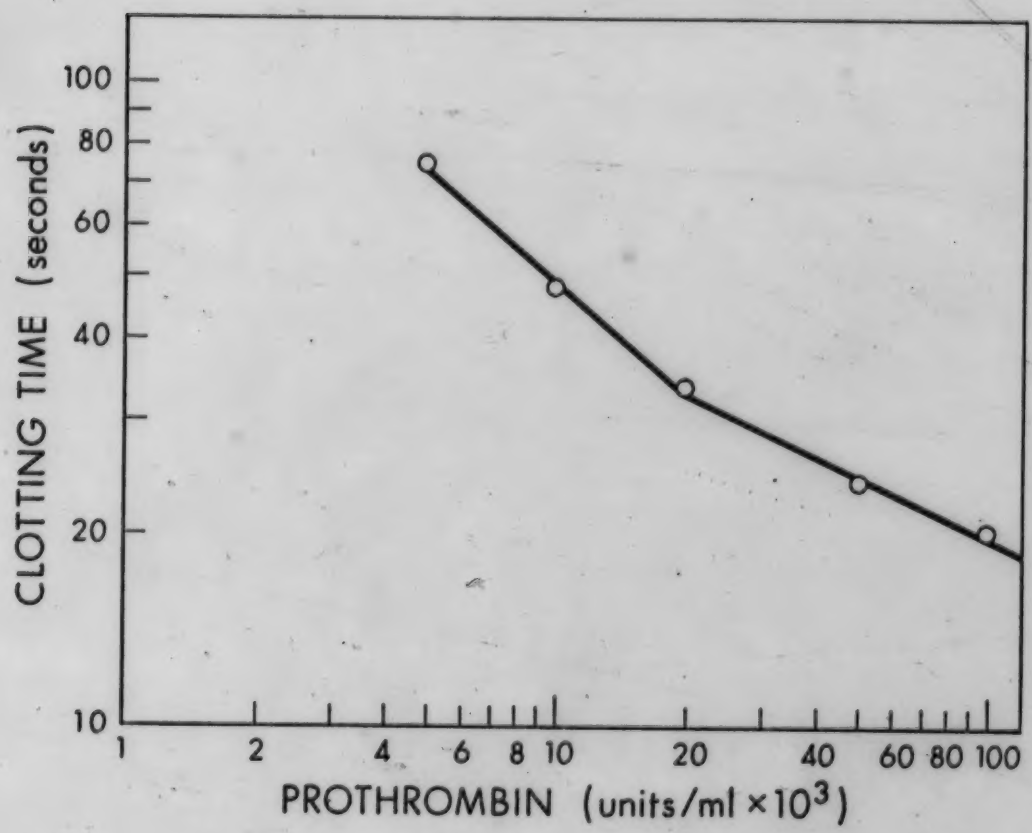


Figure 3. Calibration curve for the one-stage prothrombin assay of Hjört et al. (1955). The clotting times of dilutions of standard normal plasma are plotted. One unit of prothrombin is defined as that present in 1 ml of normal human plasma.

CHAPTER III

PROTHROMBIN-Ca^{++}-PHOSPHOLIPID COMPLEXES

A. Prothrombin characterization

1. Sephadex chromatography: The last stage of prothrombin purification is a Sephadex G-200 chromatography. The elution profile of the prothrombin used in the subsequent experiments is shown in Figure 4. The prothrombin was almost pure before chromatography and most of the protein eluted as a single peak. The peak fractions were pooled, dialyzed and stored as described in Methods. A_{280} of this material was 1.50 and the prothrombin activity was 1.98 II units /ml.

2. Amino acid analysis: The amino acid composition for this preparation is given in Table III. Values from Seegers et al. (1967), Ingwall and Scheraga (1969), Cox and Hanahan (1970), and Bull (1973) are included for comparison. Agreement of the present results with those of the other workers is very good except for proline and half-cystine which are lower than those observed by the earlier workers. This is due to difficulties encountered in their determination. Half-cystine was not converted to cysteic acid with the result that the amino acid analyzer peak tracings were too shallow for the integrator to read well. The proline peak was not well resolved from the glutamic acid peak and was therefore not properly integrated.

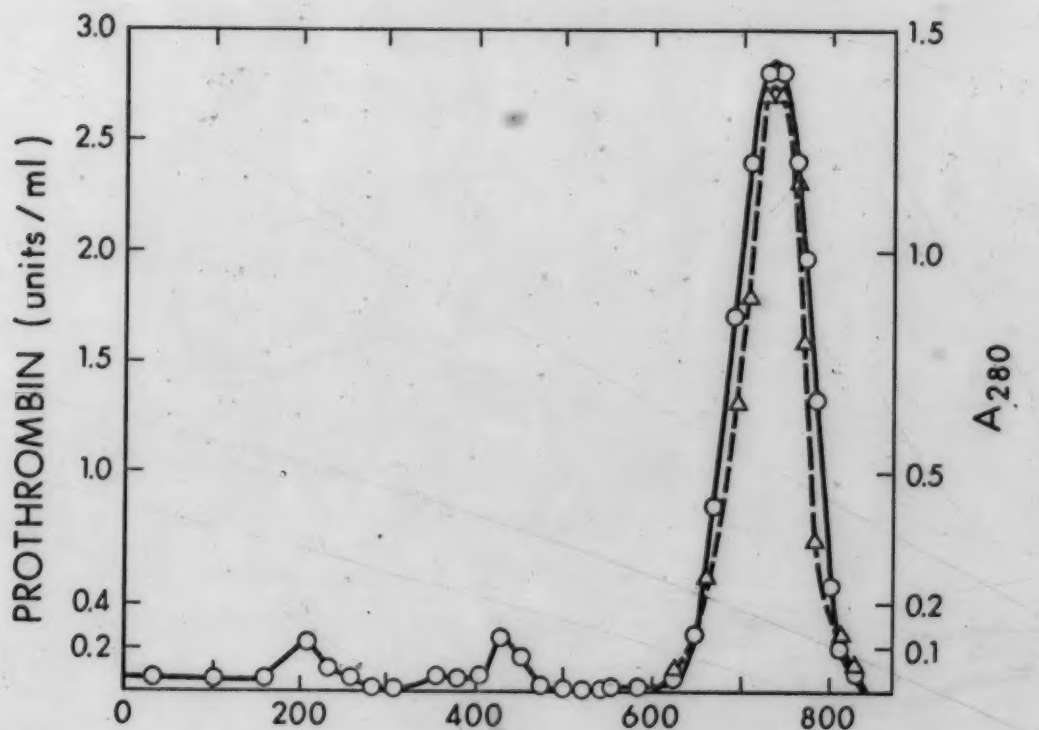


Figure 4. Sephadex G-200 chromatography of prothrombin isolated from DEAE-cellulose chromatography. Eluting solvent was Michaelis buffer pH 7.3 with 0.001 M EDTA and 10^{-4} M DFP. Prothrombin activity —△—△—△— , absorbance at 280 nm —○—○—○—

TABLE III

AMINO ACID COMPOSITION OF BOVINE PROTHROMBIN

Amino Acid	Moles/6 x 10^4 g of protein				
	1	2	3	4	5
Lys	29.0	31.4	29.0	26.5	27.6
His	8.8	9.1	8.5	10.2	7.9
Arg	39.5	36.9	43.7	32.6	33.5
Asp	57.6	55.8	55.6	55.1	54.8
Glu	68.0	67.5	66.3	67.3	67.8
Thr	28.0	26.8	26.0	27.6	25.7
Ser	34.7	39.2	31.6	36.7	33.3
Cys/2	14.7	17.2	17.0	16.3	16.3
Met	5.2	5.2	5.0	6.1	3.6
Gly	46.6	46.1	44.6	48.0	46.2
Ala	32.2	33.8	31.6	32.5	30.8
Val	32.5	30.7	32.9	34.7	31.2
Ile	18.2	16.2	17.9	18.4	19.3
Leu	42.1	42.3	41.8	41.9	35.4
Pro	27.4	29.7	31.7	32.6	29.9
Tyr	18.7	16.5	18.1	19.4	14.5
Phe	18.6	21.4	18.2	19.4	17.1
Trp	----	11.3	11.3	11.2	14.3

1 present study (Trp not determined)
2 Bull 1973
3 Cox and Hanahan 1970
4 Seegers et al. 1967
5 Ingwall and Scheraga 1969

3. Ultracentrifuge studies: Two and a half months after its preparation, a sample of prothrombin was thawed and examined in the analytical centrifuge. Photographs of the Schlieren patterns observed are shown in Figure 5. Some extra peaks are present but most of the material was in the position expected of prothrombin. Between pH 5 and 6, $S^{o}_{20,w}$ values of 4.6 S (Cox and Hanahan 1970), 4.80 S (Ingwall and Scheraga 1969), 4.84 S (Lamy and Waugh 1953), 5.22 S (Seegers 1962), and 5.3 S (Seegers et al. 1967) have been reported while at pH 7.4, a value of 5.03 S has been found (Bull 1973). The $S^{o}_{20,w}$ was not calculated here because of the uncertainty of ultracentrifuge measurements at low concentrations (Figure 6). The positive slope concentration dependence of the sedimentation coefficient at low concentrations is similar to that reported by Cox and Hanahan (1970).

4. Disc gel electrophoresis: Polyacrylamide gel electrophoresis was performed on prothrombin thawed three and a half months after preparation. Results are shown in Figure 7. Three or four faint bands are visible but the preparation is still essentially homogeneous.

5. Clotting assay: A clotting test was done to determine the effect of lyophilization of prothrombin on its activity. From the original activity of 1.98 II units/ml, 1.38 II units/ml remained after lyophilization. This represents a loss of 30% of the original activity.

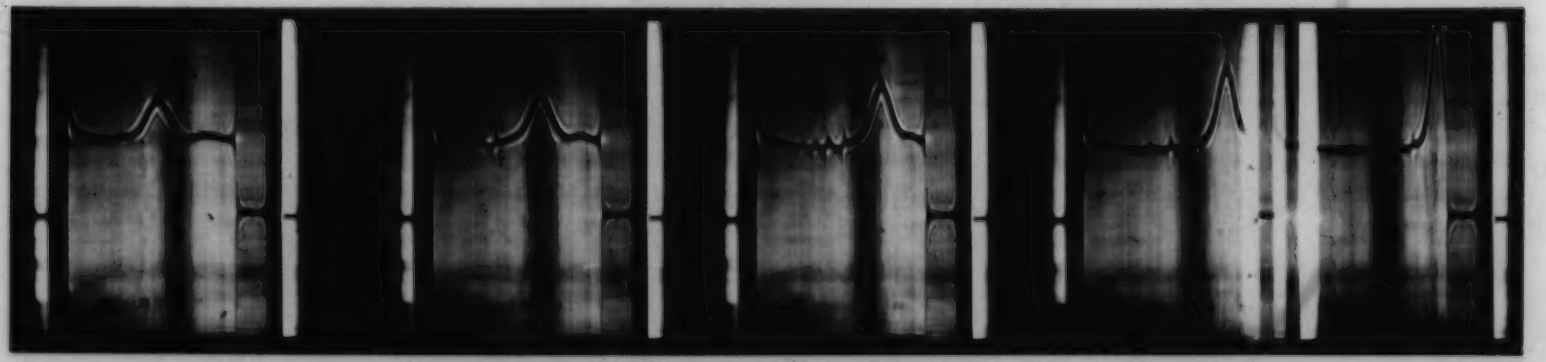


Figure 5. Schlieren patterns obtained by sedimentation velocity analytical ultracentrifugation of prothrombin. Sample concentration was 10.0 mg/ml in 0.1 M NaCl. Rotor speed was 60,190 rpm and the temperature 20°C. Photographs were taken at 16 minute intervals.

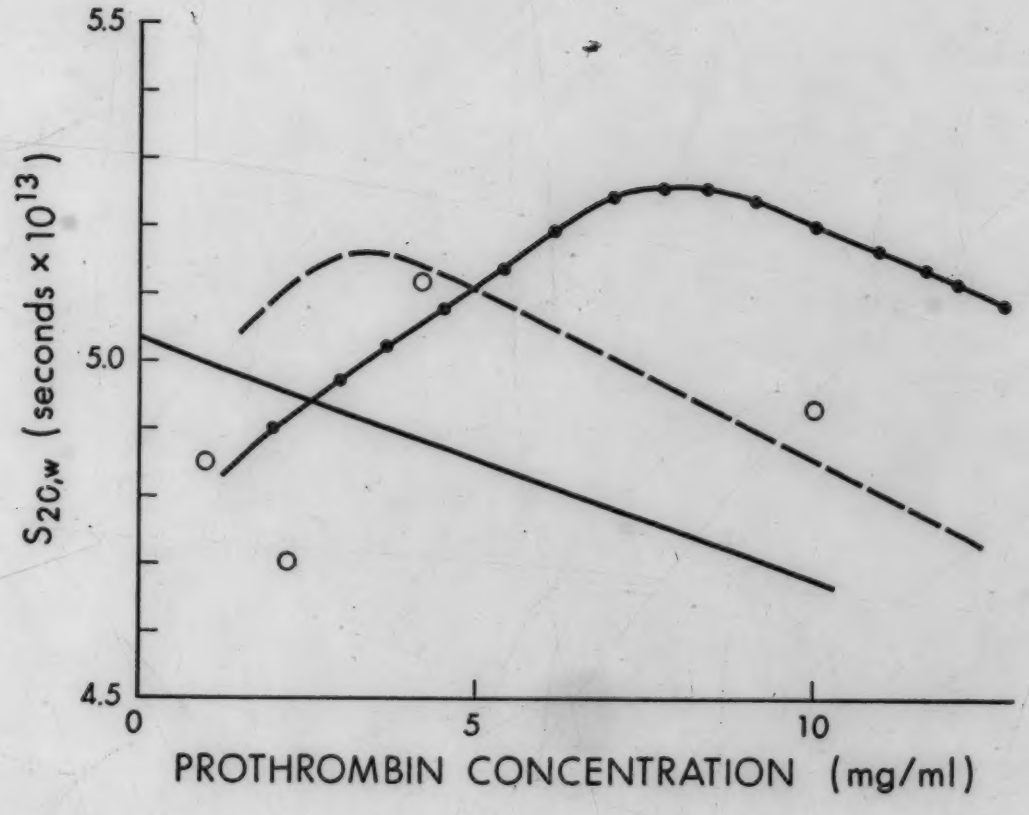


Figure 6. Concentration dependence of the sedimentation coefficient of prothrombin. Rotor speed was 60,190 rpm, temperature 20°C. Prothrombin was dissolved in 0.1 M NaCl, pH ~6.5. The curves are taken from Cox and Hanahan (1970) —●—●— at pH 5.6, — — — at pH 7.4 and from Bull (1973) ——— at pH 7.4.

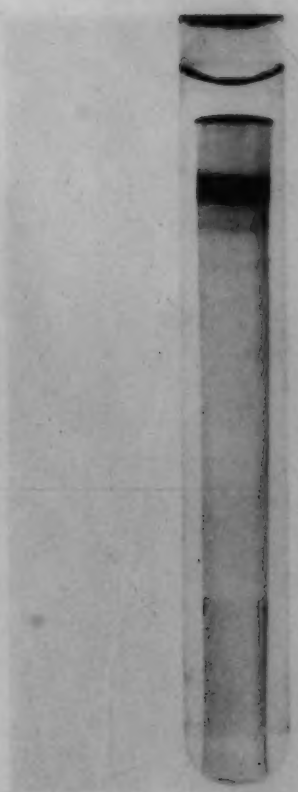


Figure 7. SDS polyacrylamide gel electrophoresis of prothrombin, according to Weber and Osborn (1969). Sample size was 10 μg. Staining was accomplished using Coomassie brilliant blue, destaining by electrophoresis in acetic acid. The anode is at the bottom.

B. Preparation procedures

Mixtures of PS:PC containing 0% PS to 80% PS (w/w) were prepared as described in Methods. Total lipid concentration was generally 12 mg/ml. Addition of 0.01 M $CaCl_2$ increased light scattering and eventually precipitated sonicated PS:PC samples. Resonication of PS:PC mixtures containing 20% PS resulted in the suspension of this material but only for a short time. Mixtures containing 40% PS with Ca^{++} were usually turbid while those with 80% PS and Ca^{++} were in particulate form.

For studies on prothrombin-lipid mixtures, the ratio of prothrombin (A_{280} units) to phospholipid (mg) was 0.76 to 1.00. This is within the range used by Bull (1973) reported as 0.76 to 1.46. At first, experiments were performed with prothrombin that was simply lyophilized. This was done in order to reduce the HDO peak in the NMR spectrum. It was realized, however, that this would produce very high salt concentrations. Therefore, a step involving dialysis against water, pH 7.1 to 7.3, was introduced. Lyophilization also introduced other difficulties. The dry prothrombin was not easily soluble. Attempts were made to solubilize the prothrombin with small amounts of D_2O before addition of the sonicated phospholipid. It again only partly dissolved. Prothrombin did not give an NMR spectrum at the concentrations used.

The effect of cytochrome c on the phospholipid NMR signals was investigated in order to ascertain the

magnitude of the change in linewidth in this simple phospholipid-protein system. In this case, Ca^{++} is not required for binding to occur.

For mixtures of 20% PS with cytochrome c, final concentrations of 3.0 to 3.6 mg/ml of cytochrome c were used. This concentration of cytochrome c was chosen to produce near equivalence of positive and negative charges. Cytochrome c has an excess of nine arginyl and lysyl residues over glutamyl and aspartyl residues (Margoliash et al. 1961). For PC and for mixtures with 40% PS, 60% PS, and 80% PS, the concentration of cytochrome c employed was from 5.9 to 6.7 mg/ml. Cytochrome c, at these concentrations, did not give a resolved NMR spectrum.

A set of spectra of 20% PS: 80% PC alone and with cytochrome c, Ca^{++}, and Ca^{++}-prothrombin are shown in Figures 8, 9, 10, and 11.

C. Results

1. Choline methyl linewidths

Substantial variation in NMR linewidths was observed in different experiments with PS:PC mixtures of the same composition. The reason for this dispersion of values is not known but may reflect different efficiencies of sonication. In order to ensure comparability, average values of the $-\overset{+}{N}(CH_3)_3$ linewidths (at a chemical shift of

Figure 8. The NMR spectrum of a mixture of 20% PS-80% PC in D_2O. Phospholipid concentrations were 10.6 mg PS, 42.6 mg PC in 4.4 ml; pH 7.0; temperature 29-31° C. Shown is a 345 Hz section of a 500 Hz spectrum. Chemical shift values refer to position downfield from TMS.

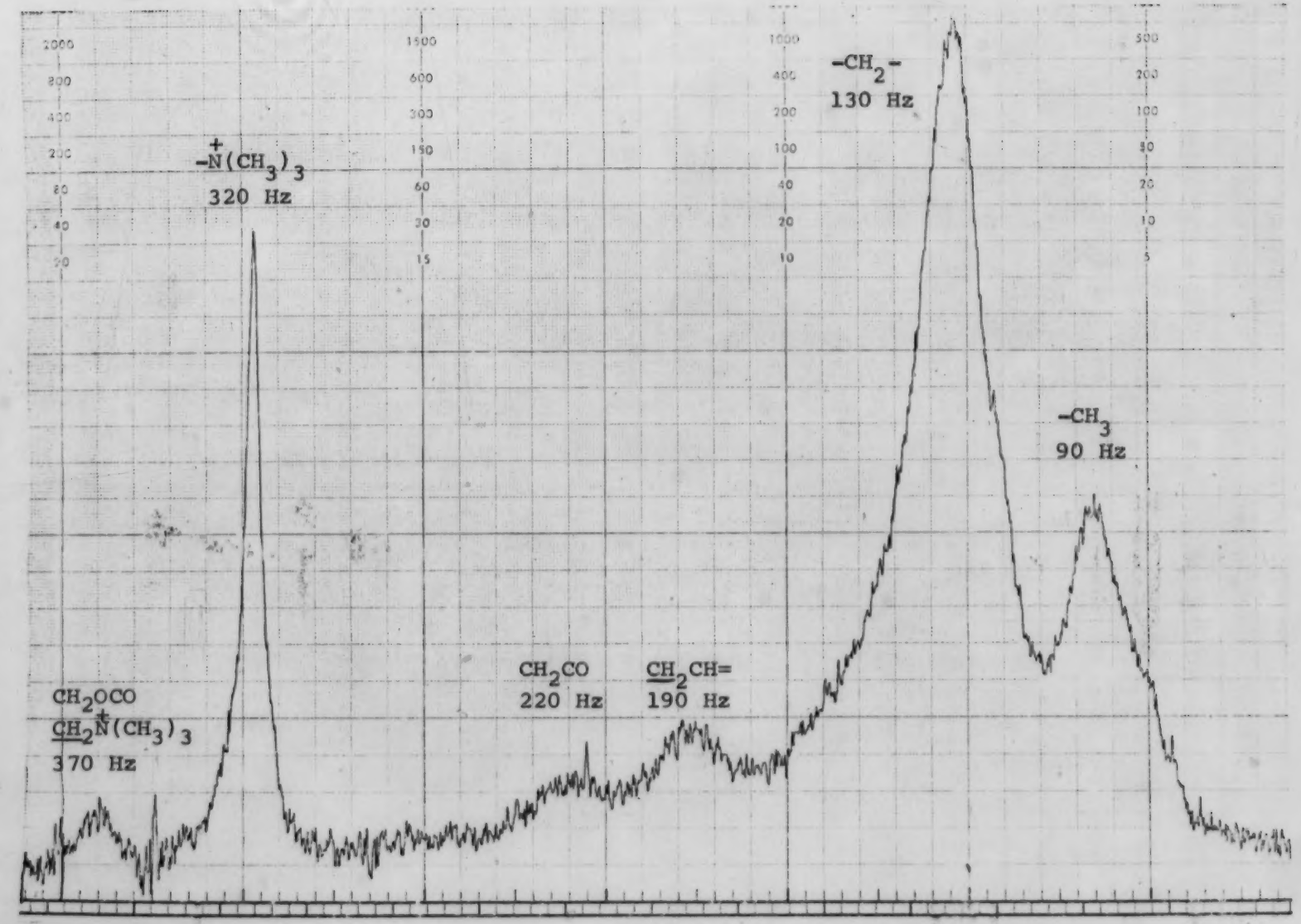

$-\overset{+}{N}(CH_3)_3$
320 Hz
$-CH_2-$
130 Hz
$-CH_3$
90 Hz
CH_2CO
220 Hz
$\underline{CH_2}CH=$
190 Hz
CH_2OCO
$\underline{CH_2}\overset{+}{N}(CH_3)_3$
370 Hz

Figure 9. The NMR spectrum of a mixture of 20% PS-80% PC in D_2O with cytochrome c. The phospholipid sample is the same as in Figure 8. Measurement was on a 0.52 ml sample containing 5.0 mg PC, 1.2 mg PS and 1.9 mg cytochrome c.

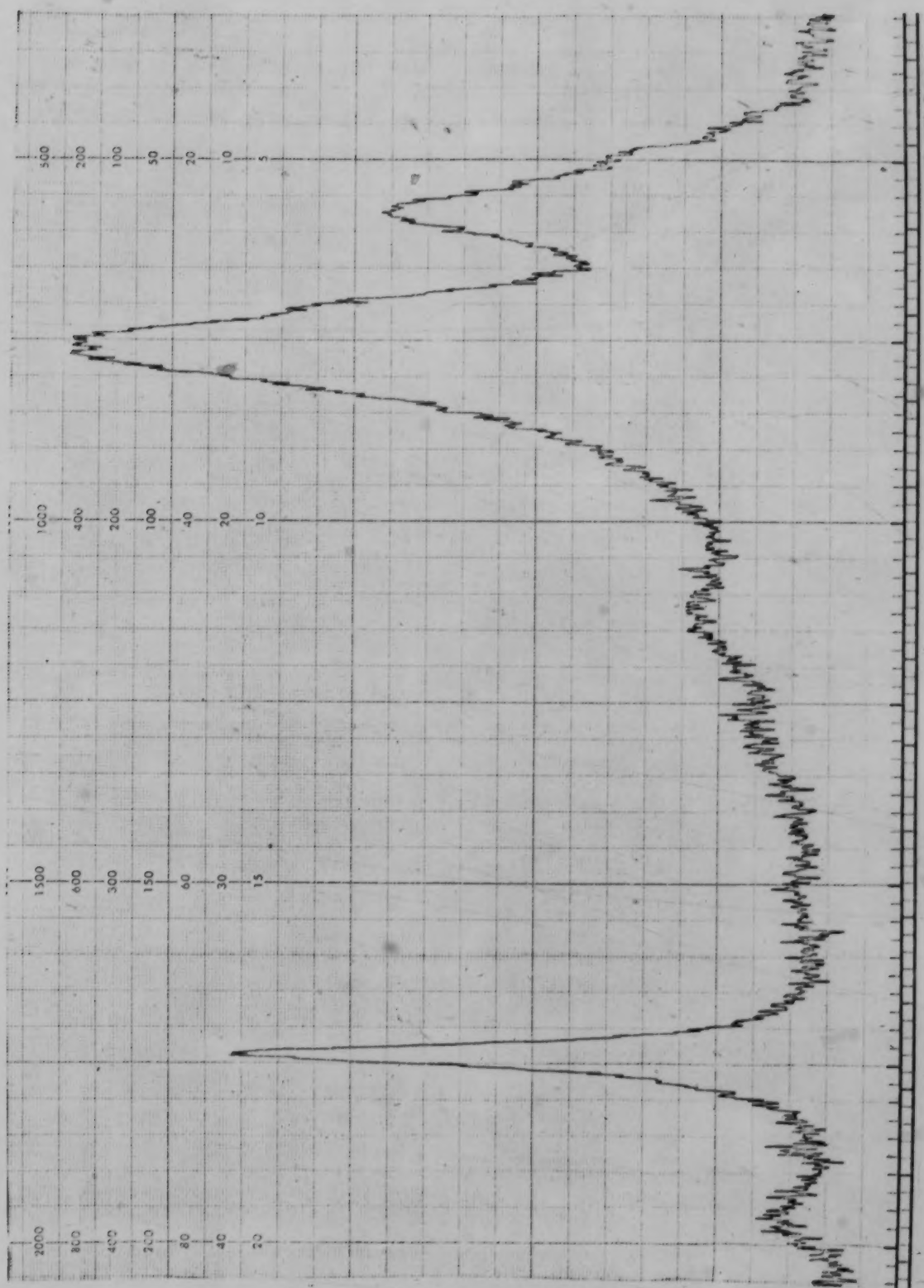

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Figure 10. The NMR spectrum of a mixture of 20% PS-80% PC in D_2O with Ca^{++}. The phospholipid sample is the same as in Figure 8. The total phospholipid concentration was 12 mg/ml, the Ca^{++} concentration 0.01 M; pH 7.4.

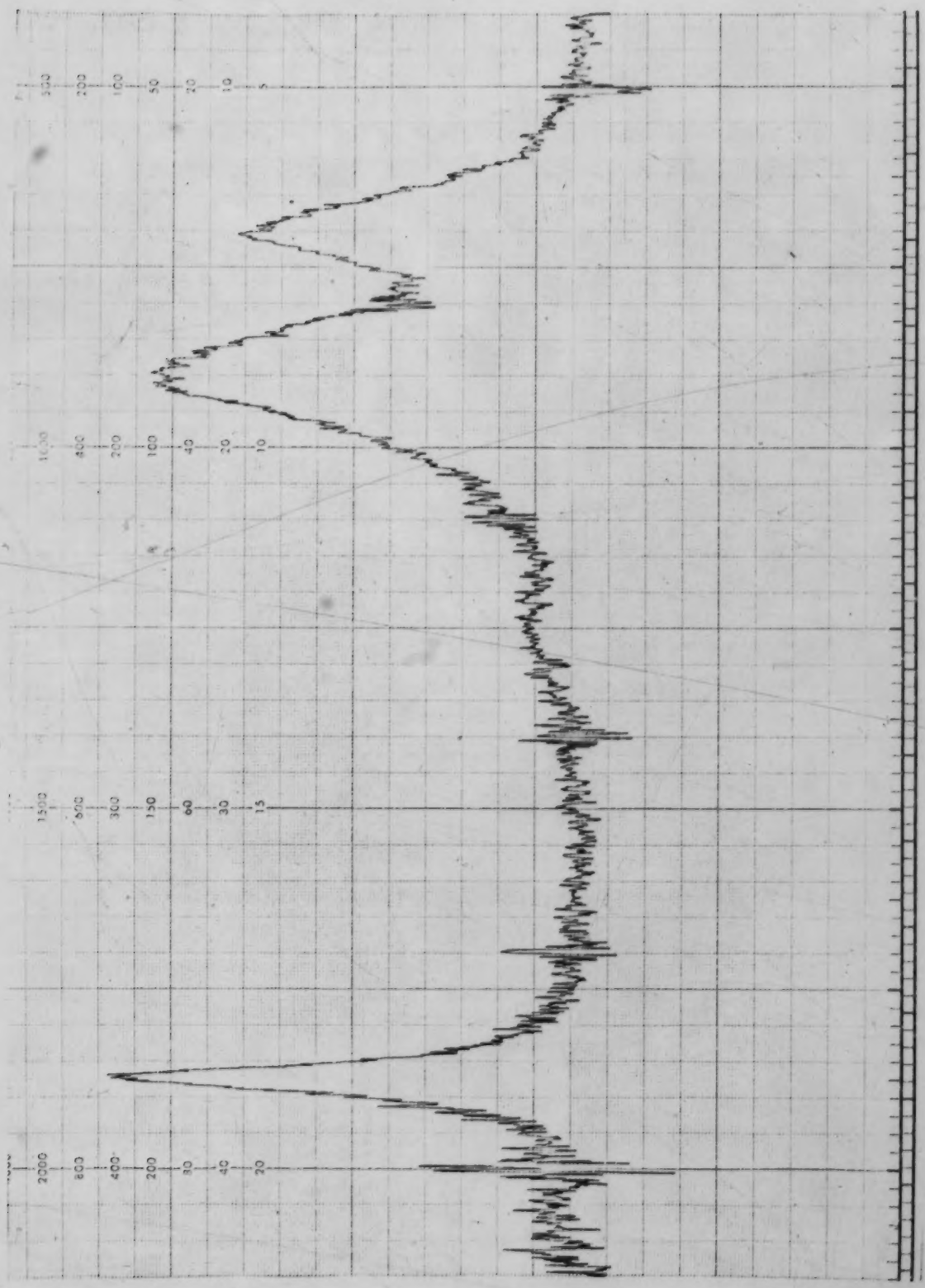
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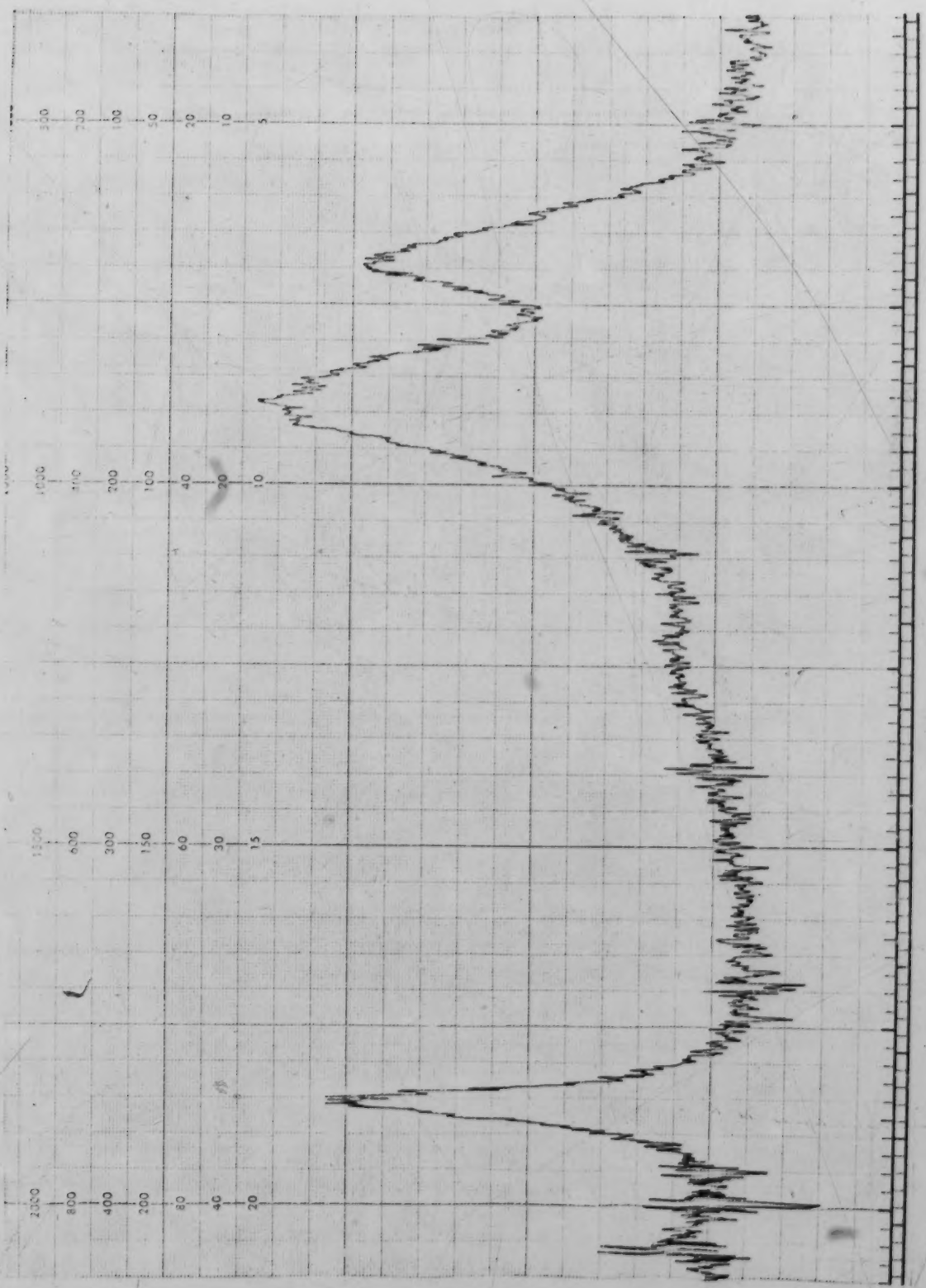
Figure 11. The NMR spectrum of a mixture of 20% PS-80% PC in D_2O with Ca^{++} and prothrombin. To a 0.52 ml aliquot of the sample of Figure 10, containing 6.2 mg of phospholipid, 6 A_{280} units of prothrombin were added.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40
20

325 Hz downfield from TMS) were calculated at each PS:PC composition for the phospholipid mixtures in the absence of any Ca^{++} or protein. For experiments with Ca^{++}, prothrombin-Ca^{++} or cytochrome c added, the observed linewidths were multiplied by the factor needed to bring the corresponding simple phospholipid samples to these average figures. This procedure reduced the scatter of points. Results obtained by this technique were averaged and the average values are plotted in Figure 12. The decrease in the value for the linewidths of the phospholipid mixtures alone is not significant considering the experimental error involved. When cytochrome c was added to mixtures containing PS, the linewidth increased substantially but this was not so when PC was used. When Ca^{++} was added, the increase was even greater than when cytochrome c was added but again there was no effect on the linewidth for PC samples. This is as expected, since Ca^{++} does not bind significantly to PC in water (Shah and Schulman 1965, 1967, Kimizuka et al. 1967, Papahadjopoulos 1968). The addition of prothrombin to phospholipid-Ca^{++} samples caused a slight further increase in linewidth. The values for mixtures containing 60% PS and Ca^{++} are only approximate since a loss of signal intensity made an estimate of half-height width very difficult.

2. Methylene resonance splitting

Because of the splitting of the methylene NMR resonance (approximately 128 Hz downfield from TMS), it

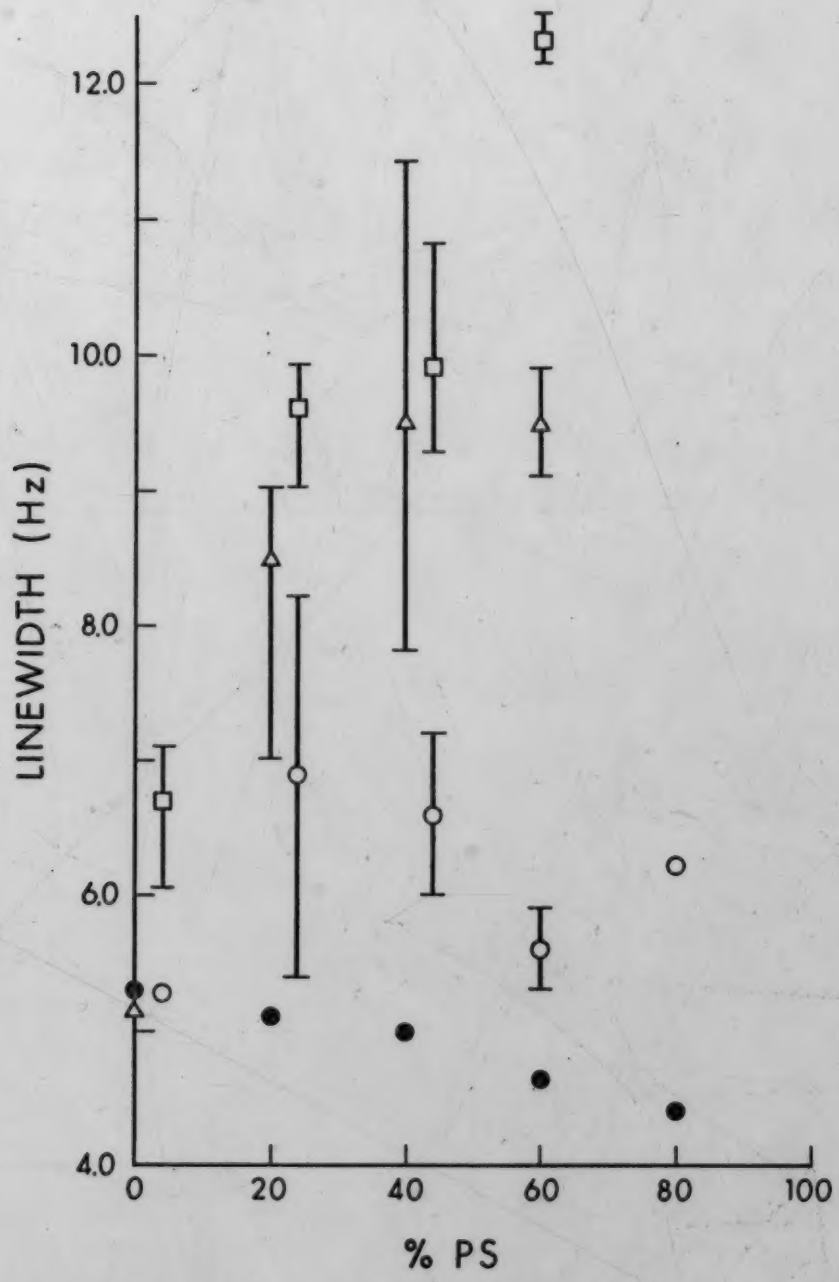


Figure 12. $-\overset{+}{N}(CH_3)_3$ linewidths adjusted to average values of the linewidth of phospholipids without additions. PC values are the average of 2 determinations; 20% PS of 5-10; 40% PS of 3-4; 60% PS of 2; and 80% PS is a single determination. ● average value of phospholipid alone; ○ PL with cytochrome c; △ PL with Ca^{++}; □ PL-Ca^{++}-prothrombin. Lines represent scatter. The values of cytochrome c and prothrombin-Ca^{++}-phospholipid are shifted to the right for ease of reading.

was not possible to calculate linewidths. Rather, the relative peak heights were investigated. The results are shown in Figures 13, 14, 15, and 16. The chemical shift is referred upfield to the $-\overset{+}{N}(CH_3)_3$ resonance. No splitting was observed with pure PC or with mixtures containing 20% PS (Figure 13). With 40% PS content, a peak appeared 207 Hz upfield from $-\overset{+}{N}(CH_3)_3$. As the relative PS concentration increased, this peak increased concomitantly.

When cytochrome c was added to a mixture containing 20% PS, the methylene resonance appeared to be split. At higher PS concentrations, the downfield peak was most affected. At 40% PS, the relative peak heights became equal. At 60% PS, the downfield component had decreased to a shoulder and at 80% PS, a single asymmetrical peak was observed.

Ca^{++} induced similar though more pronounced effects. At 20% PS, the peak was broader than either 20% PS alone or 20% PS with cytochrome c. Already at 40% PS, only an asymmetrical peak appeared while at 60% PS and 80% PS, the peak originating from the upfield component was symmetrical.

Prothrombin had only a marginal broadening effect on the spectra of phospholipid-Ca^{++} samples. This can be seen by comparing Figures 15 and 16.

3. Methyl resonance splitting

In the case of the $-CH_3$ resonances (chemical shift approximately 90 Hz downfield from TMS) up to three

Figure 13. The $-CH_2-$ region of the NMR spectrum of mixtures of PS and PC. Chemical shift refers to position upfield from the $-N(CH_3)_3$ signal.
a) pure PC: 12 mg/ml, pH 7.8
b) 20% PS: 10.6 mg PS, 42.6 mg PC in 4.4 ml, pH 7.0 (same phospholipid sample as shown in Figures 8-11)
c) 40% PS: 21.3 mg PS, 32.1 mg PC in 4.0 ml, pH 7.7
d) 60% PS: 25.1 mg PS, 16.6 mg PC in 3.4 ml, pH 6.8
e) 80% PS: 33.6 mg PS, 8.3 mg PC in 3.4 ml, pH 7.8

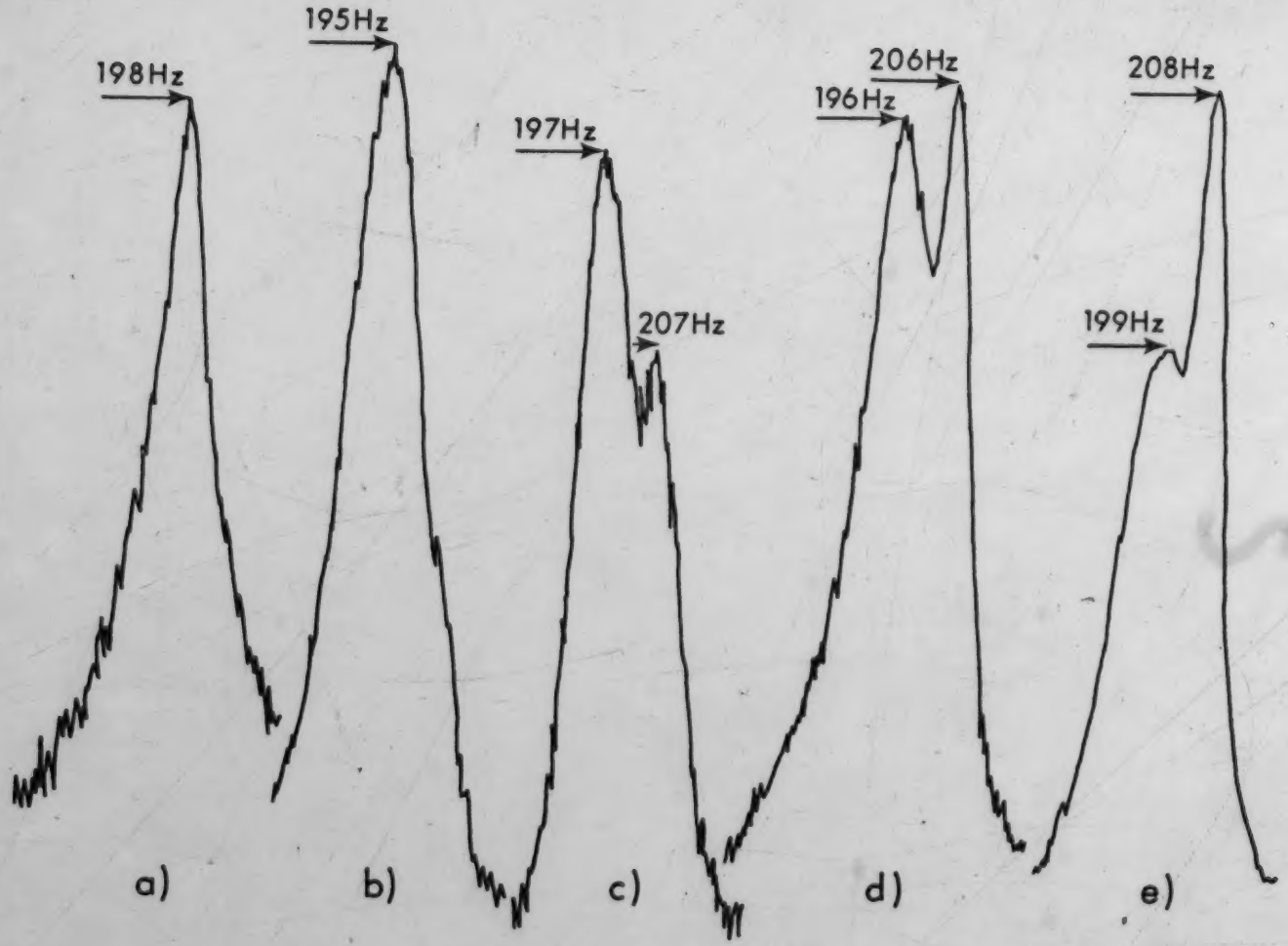

198Hz
195Hz
197Hz
207Hz
196Hz
206Hz
208Hz
199Hz
a)
b)
c)
d)
e)

Figure 14. The $-CH_2-$ region of the NMR spectrum of mixtures of PS and PC with cytochrome c. Chemical shift refers to position upfield from the $-N(CH_3)_3$ signal. Phospholipid samples are the same as those in Figure 13(exc. c)

a) pure PC: 5.9 mg PC, 2.9 mg cytochrome c in 0.49 ml
b) 20% PS: 5.0 mg PC, 1.2 mg PS, 1.9 mg cytochrome c in 0.52 ml
c) 40% PS: 4.1 mg PC, 2.7 mg PS, 3.0 mg cytochrome c in 0.50 ml
d) 60% PS: 2.4 mg PC, 3.7 mg PS, 3.3 mg cytochrome c in 0.52 ml pH 7.6 (lower curve 2.5 mg PC, 3.9 mg PS, 3.0 mg cytochrome c in 0.50 ml pH 7.4)
e) 80% PS: 1.2 mg PC, 4.9 mg PS, ~3.0 mg cytochrome c in 0.50 ml

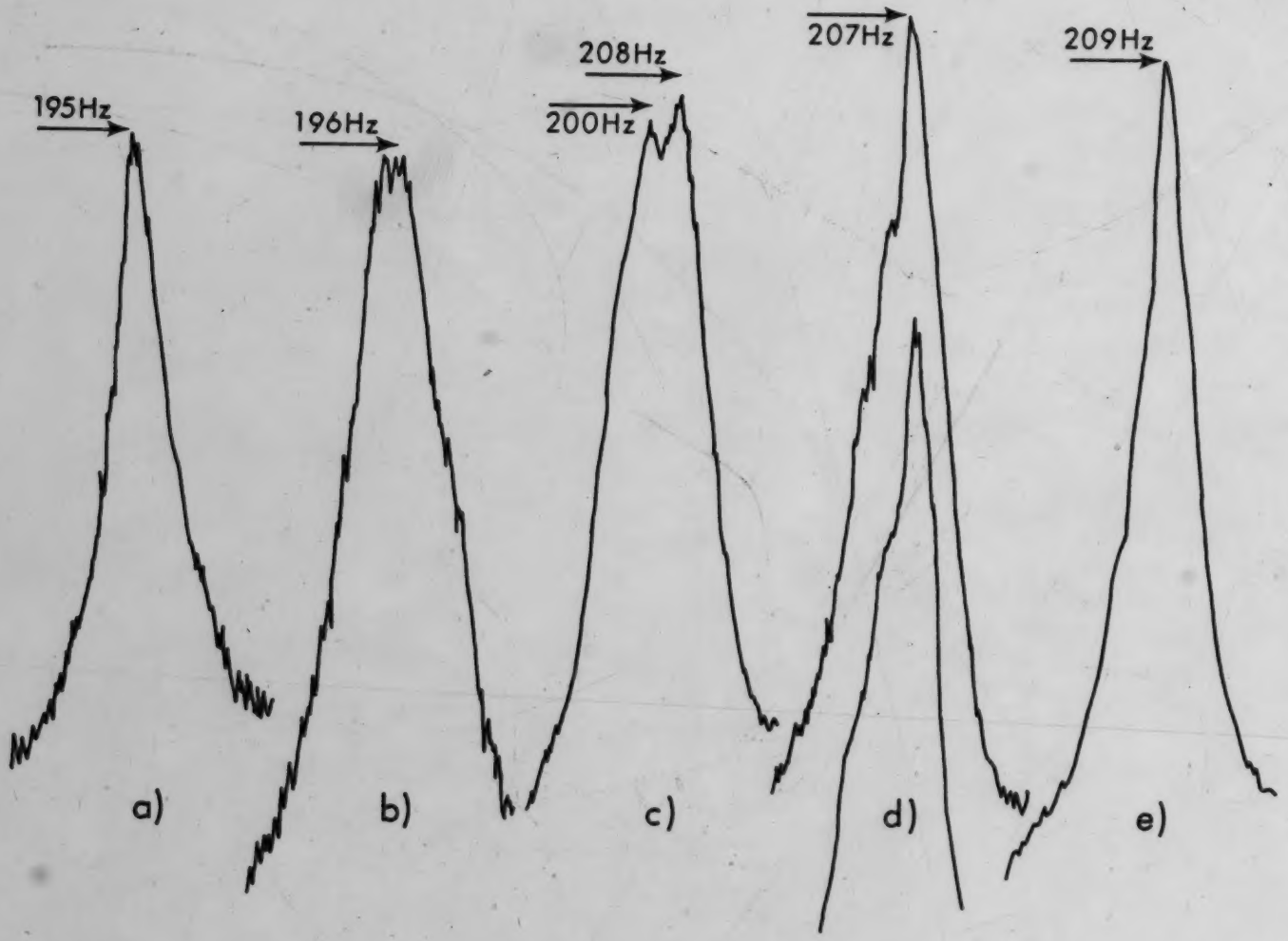

195Hz
196Hz
208Hz
200Hz
207Hz
209Hz
a)
b)
c)
d)
e)

Figure 15. The $-CH_2-$ region of the NMR spectrum of mixtures of PS and PC with Ca^{++}. Chemical shift refers to position upfield from the $-N(CH_3)_3$ signal. Phospholipid samples are the same as those in Figure 14.

a) pure PC: lipid concentration 12 mg/ml, pH 7.5
b) 20% PS: lipid concentration 12 mg/ml, pH 7.4
c) 40% PS: lipid concentration 13.2 mg/ml, pH 8.6
d) 60% PS: lipid concentration 12 mg/ml, pH 7.9
e) 80% PS: lipid concentration 12 mg/ml

In all cases, Ca^{++} concentration was 0.01 M.

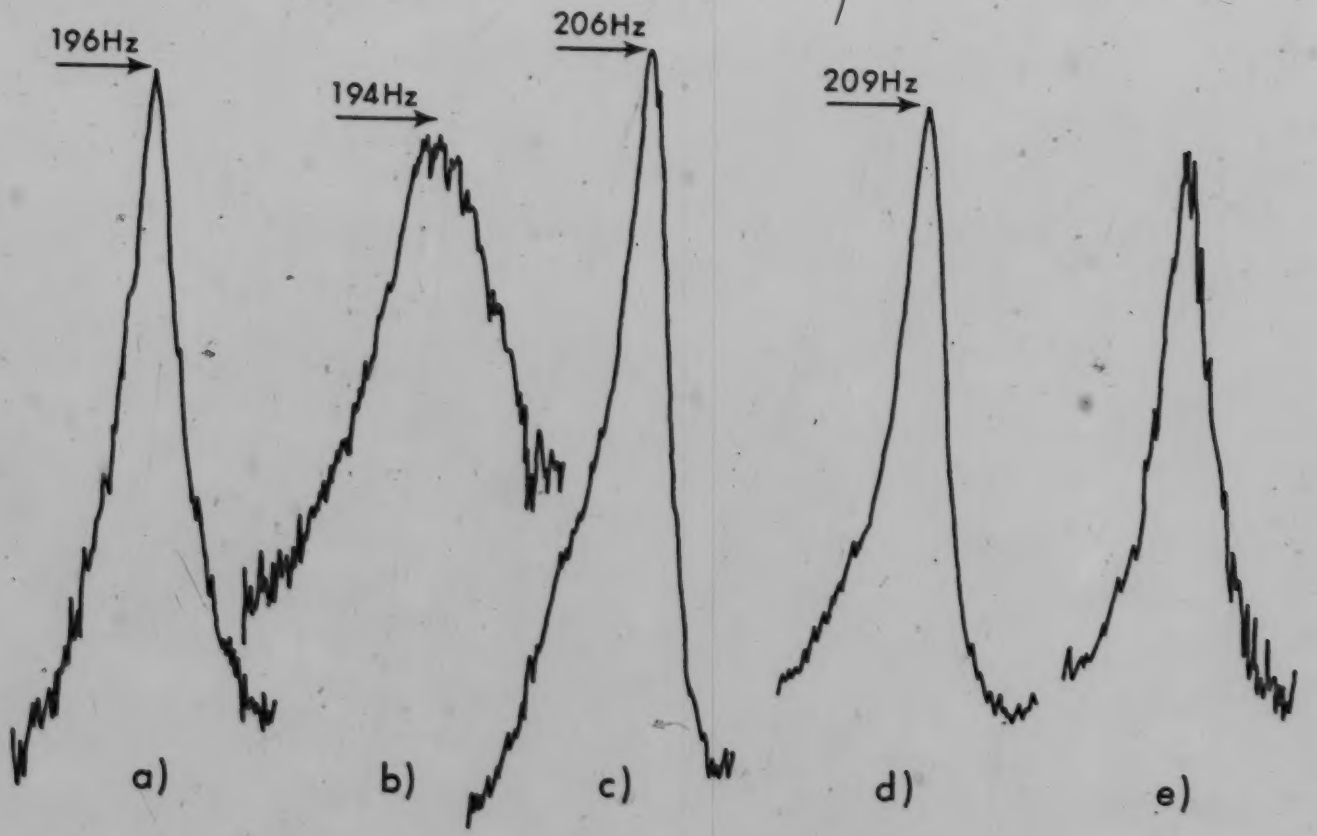
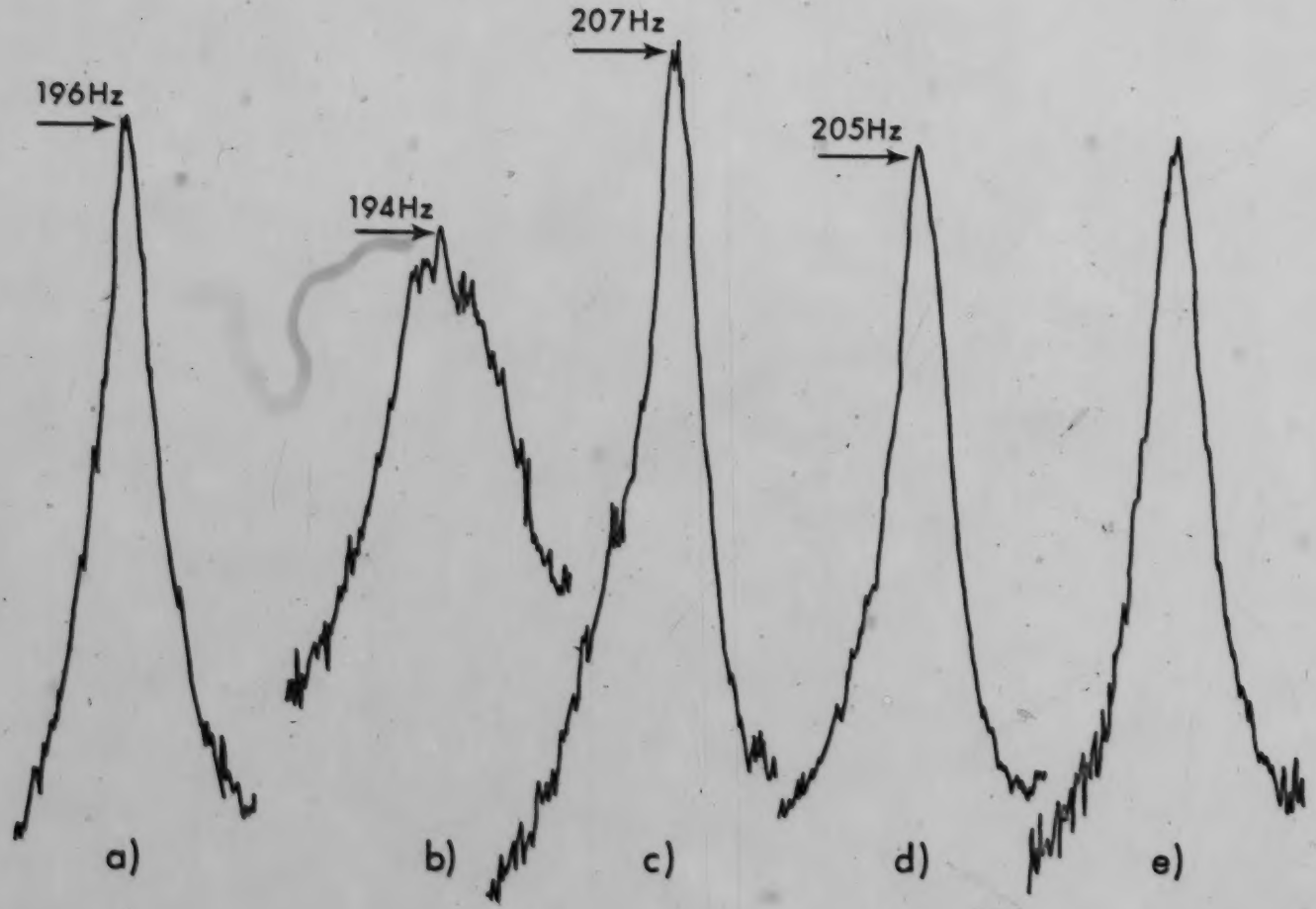

196Hz
194Hz
206Hz
209Hz
a)
b)
c)
d)
e)

Figure 16. The $-CH_2-$ region of the NMR spectrum of mixtures of PS and PC with Ca^{++} and prothrombin (Factor II). Ca^{++} concentration was 0.01 M. Phospholipid samples were those in Figure 15. Chemical shift refers to position upfield from the $-N(CH_3)_3$ signal.

a) pure PC: 6.0 mg lipid with 6 A_{280} units of II in 0.5 ml
b) 20% PS: 6.2 mg lipid with 6 A_{280} units of II in 0.52 ml
c) 40% PS: 6.6 mg lipid with 6 A_{280} units of II in 0.5 ml
d) 60% PS: ~6.0 mg lipid with 6 A_{280} units of II in 0.5 ml
e) 80% PS: ~6.0 mg lipid with 6 A_{280} units of II in 0.5 ml


196Hz
194Hz
207Hz
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a)
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components were found (Figures 17 and 18). Pure PC and mixtures containing 20% PS exhibited a single peak while at 40% PS and higher, two additional peaks appeared upfield. The downfield peak decreased rapidly on going to 60% PS and 80% PS while the middle peak exhibited a slower decline.

With cytochrome c, the relative magnitude of both the downfield and the central resonances was lessened so that the downfield peak had disappeared at 60% PS and the middle peak only appeared as a shoulder at 80% PS. After Ca^{++} addition, the effect was even more dramatic. The downfield component appeared as a shoulder at 40% PS and the central component as a shoulder at 60% PS. At 80% PS, only a single peak was seen. Prothrombin-Ca^{++}-phospholipid samples were again similar to the Ca^{++}-phospholipid complexes although they showed slightly more broadening of the downfield and middle resonance lines. At 20% PS, prothrombin separated the peak into two components.

4. Discussion

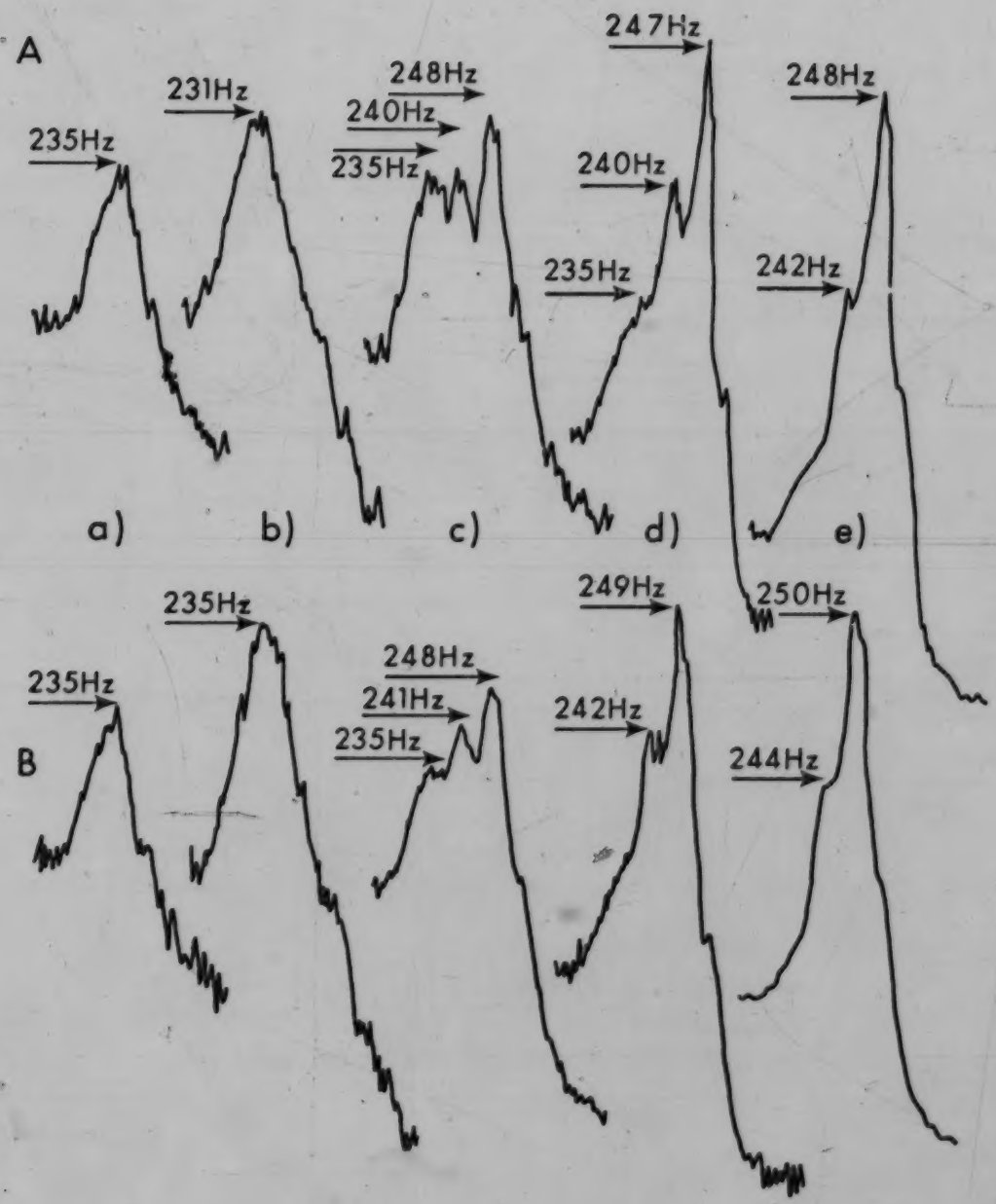
These results could be explained if it were assumed that:

a) in the absence of Ca^{++}, addition of PS to PC up to 30% PS resulted in random mixing of the two species;

b) at higher PS concentrations, the additional PS was sequestered so that two phases existed, one a mixed phase of PS + PC (molar ratio 1:3 to 1:4), the other a PS-rich phase;

Figure 17. A. The $-CH_2$ region of the NMR spectrum of mixtures of PS and PC. Chemical shift refers to position upfield from the $-N(CH_3)_3$ signal. Experimental conditions are the same as those described in Figure 13.
a) pure PC
b) 20% PS
c) 40% PS
d) 60% PS
e) 80% PS

B. The $-CH_2$ region of the NMR spectrum of mixtures of PS and PC with cytochrome c. Chemical shift refers to position upfield from the $-N(CH_3)_3$ signal. Experimental conditions are the same as those described in Figure 14.
a) pure PC
b) 20% PS
c) 40% PS
d) 60% PS
e) 80% PS


A
235Hz
231Hz
248Hz
240Hz
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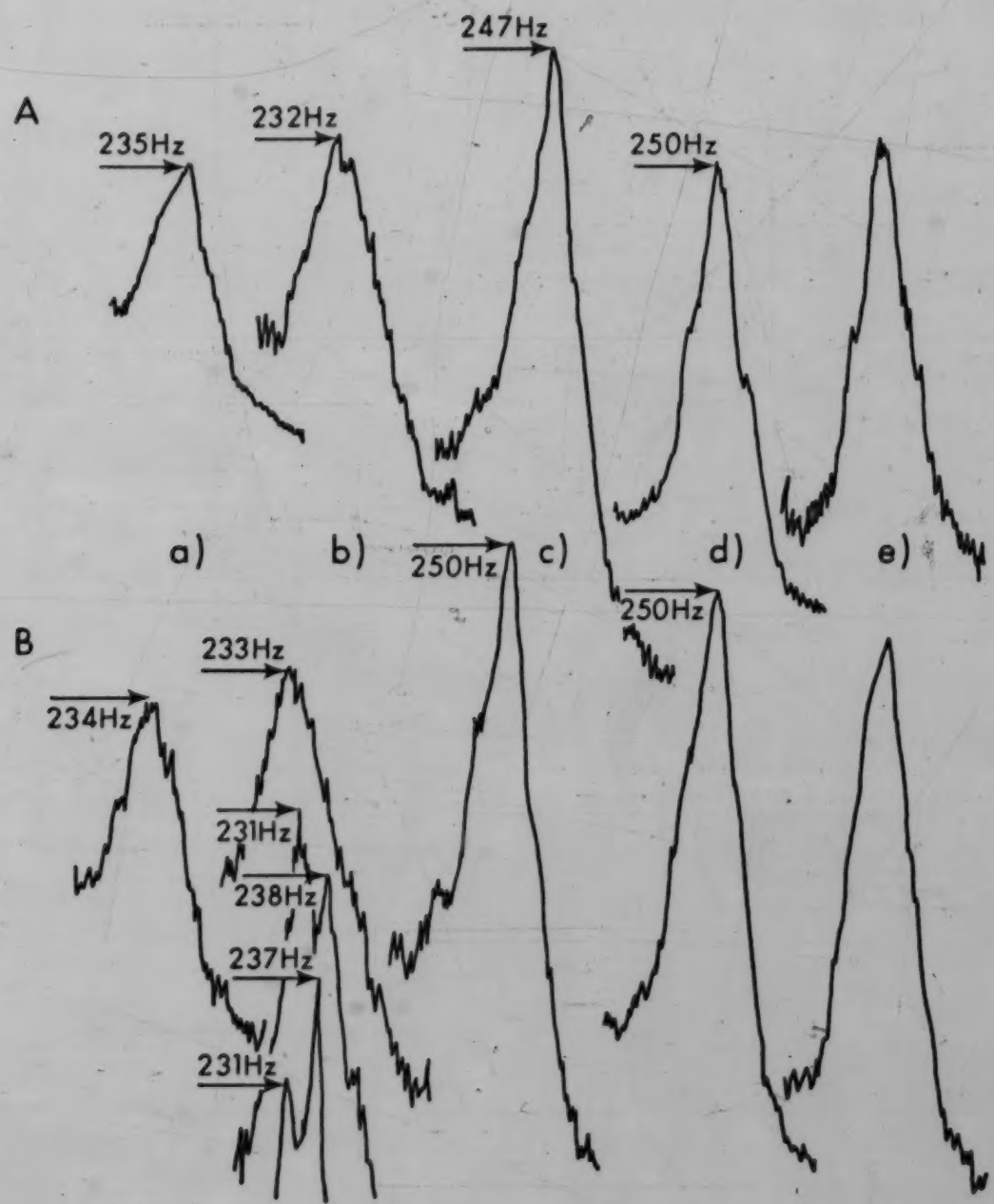
240Hz
235Hz
248Hz
242Hz
a)
b)
c)
d)
e)
B
235Hz
235Hz
248Hz
241Hz
235Hz
249Hz
242Hz
250Hz
244Hz

Figure 18. A. The $-CH_3$ region of the NMR spectrum of mixtures of PS and PC with Ca^{++} (0.01 M). Chemical shift refers to position upfield from the $-N(CH_3)_3$ signal. Experimental conditions are those described in Figure 15.

a) pure PC
b) 20% PS
c) 40% PS
d) 60% PS
e) 80% PS

B. The $-CH_3$ region of the NMR spectrum of mixtures of PS and PC with 0.01 M Ca^{++} and prothrombin. Chemical shift refers to position upfield from the $-N(CH_3)_3$ signal. Experimental conditions are those described in Figure 16.

a) pure PC
b) 20% PS (middle and lower curves are different samples)
c) 40% PS
d) 60% PS
e) 80% PS


A
235Hz
232Hz
247Hz
250Hz
a)
b)
c)
d)
e)
B
234Hz
233Hz
231Hz
238Hz
237Hz
231Hz
250Hz
250Hz

c) all the $-CH_2-$ protons in the PS:PC phase (whether protons of PS or PC) gave rise to the low-field component (195-198 Hz above the $-\overset{+}{N}(CH_3)_3$ resonance) while all the $-CH_2-$ protons in the PS-rich phase gave rise to the high-field component (206-209 Hz above the $-\overset{+}{N}(CH_3)_3$ signal).

Consequently, we would see one component at 0% and 20% PS. Above this PS composition, the high-field component would appear and subsequently, the relative intensities of the two components would depend on the ratio of PS to PC.

The addition of Ca^{++} to the 20% PS sample caused broadening of the $-\overset{+}{N}(CH_3)_3$ line and the single $-CH_2-$ signal. With mixtures containing a higher proportion of PS, the effect of Ca^{++} was to broaden the downfield component of the $-CH_2-$ resonance. This behaviour presumably occurred because Ca^{++} forms a di PS-Ca^{++} complex within the mixed phase (Figure 19), increasing the degree of order of both the hydrocarbon chains and the polar groups. A lattice structure of PS and Ca^{++} has been proposed for bilayer membranes by Papahadjopoulos (1968) (see Chapter V).

As the proportion of PS was increased, the low-field component remained very wide while the intensity of the high-field component increased. To interpret this, one may propose that Ca^{++} is unable to immobilize molecules in the PS-rich phase. The ordering of PS molecules by Ca^{++} may, for example be inconsistent with the requirement for curvature in the small vesicles. This reasoning is

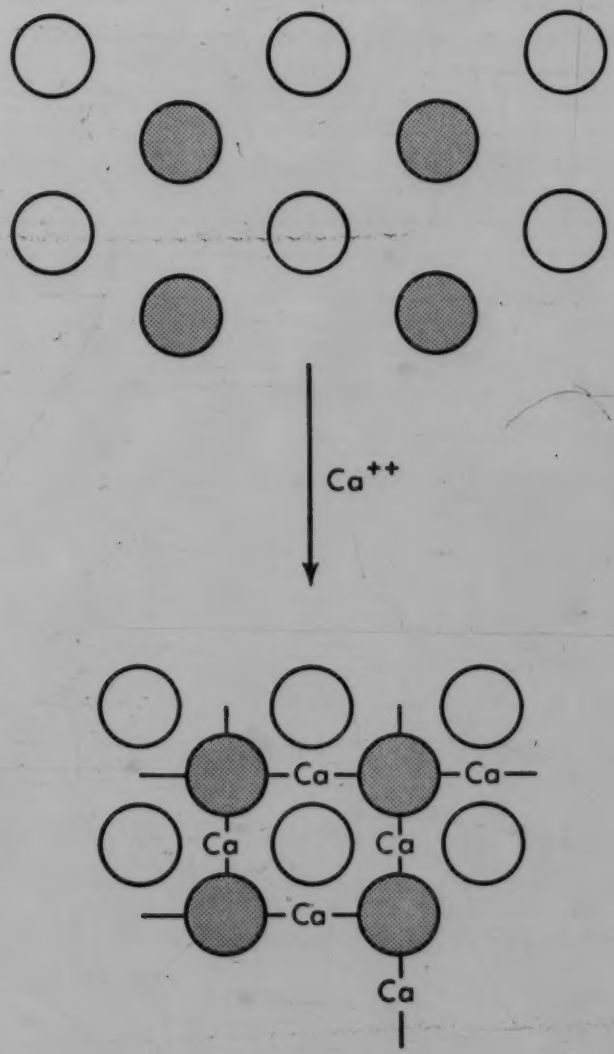


Figure 19. Diagrammatic representation of the proposed interaction between Ca^{++} and a mixed PS:PC phase.

○ PC; ◉ PS

supported by the fact that the $-\overset{+}{N}(CH_3)_3$ linewidth was broadened dramatically by Ca^{++} up to 20-40% PS but above this, in the region 40 to 60% PS, the width remained approximately constant.

Prothrombin added to mixtures containing 20% PS and Ca^{++} produced somewhat more broadening of the $-CH_2-$ and $-\overset{+}{N}(CH_3)_3$ lines than did Ca^{++} alone. Since the protein has 3 or 4 high affinity Ca^{++} binding sites (Barton 1971, Nelsestuen and Suttie 1972a, Stenflo and Ganrot 1973), it can act as a multidentate ligand stabilizing the lattice arrangement and further immobilizing the molecules of PS and PC. However, such an effect of prothrombin was slight.

Prothrombin did not appear to significantly affect the relative distribution of the lipid phases as evidenced by comparison of the spectra of the 40% PS and 60% PS mixtures in Figures 16 and 17. The small effect of prothrombin on the $-\overset{+}{N}(CH_3)_3$ signal of PC-Ca^{++} does not appear to correlate with the lack of complex formation (Bull 1973).

The effect of cytochrome c, a polybasic protein, could be explained in two ways. It could facilitate somewhat the sequestration of PS into the PS-rich phase, leading to a loss of the low-field $-CH_2-$ component intensity in favour of the dominant high-field component. Alternatively, it could interact almost exclusively with the mixed PS:PC phase and cause a broadening of its resonances.

Since the cytochrome c was added after sonication, it should interact only with the outer layer of the vesicles. Berden et al. (1975) have reported that PS is preferentially located on the inside of the vesicles. On the basis of the binding experiments of Kimelberg and Papahadjopoulos (1971, 1971a), Butler et al. (1973) and Van and Griffiths (1975), to be discussed more fully in Chapter V, the second explanation would appear more likely. According to Jacobson and Papahadjopoulos (1975), cytochrome c does not effect a separation of phospholipids when added to 66% beef brain PS - 34% dipalmitoyl PC.

The resonances of the mixed PS:PC phase were not broadened as much by cytochrome c as they were by Ca^{++}. As can be seen by the lack of effect of cytochrome c on the linewidth of the $-\overset{+}{N}(CH_3)_3$ resonance of pure PC, cytochrome c does not interact at all with PC.

CHAPTER IV

A RE-EVALUATION OF THE PHOSPHOLIPID COMPONENT

Nine and a half months after the end of the experiments presented in the previous chapter, a sample of the phosphatidyl serine (PS-1) used in those studies, without added PC, was examined by NMR. The spectrum exhibited two $-CH_2-$ peaks and at least two $-CH_3$ peaks. The height of the downfield $-CH_2-$ component was slightly greater than that of the upfield component. This was not consistent with the trend established with PS:PC mixtures on increasing PS content. Furthermore, Hauser and Phillips (1973) had meanwhile reported an NMR spectrum of bovine brain PS exhibiting a single $-CH_2-$ peak. Alternative explanations of the two $-CH_2-$ lines were therefore sought. Since bovine brain PS contains a high proportion of polyunsaturated fatty acids, it is rather susceptible to oxidation. One of the two component lines might therefore have originated from oxidation products. The presence of contaminants in the preparation of PS could also produce artifacts of this kind.

A new preparation of PS (PS-2) was carried out. It was judged pure in three TLC systems (Figure 20). The NMR spectrum exhibited no splitting of the $-CH_2-$ peak either for pure PS or for mixtures of PS and PC containing 10% to 40% PS. Linewidth results using this PS preparation are given in Table IV. The ordering effect of Ca^{++} was

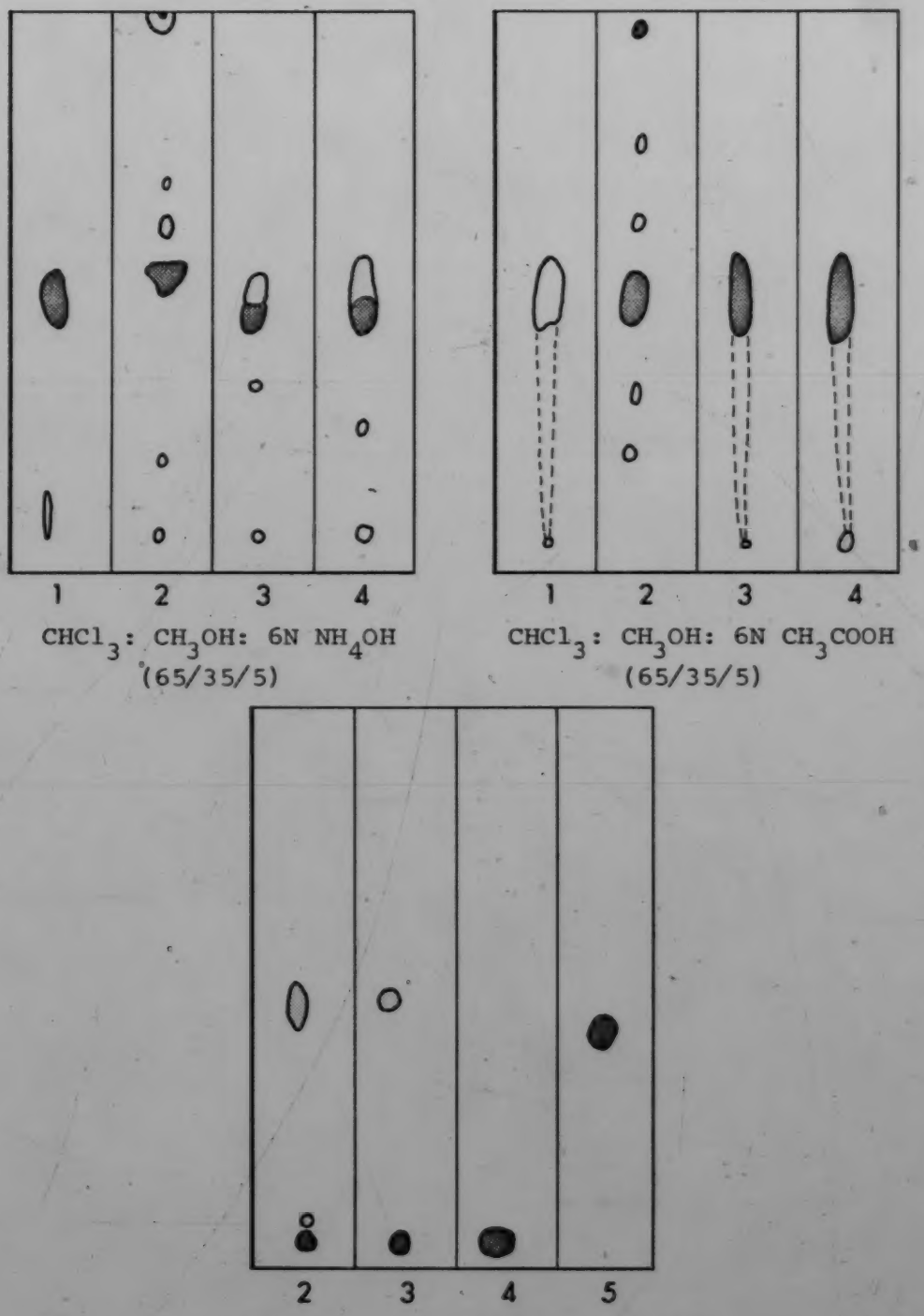


Figure 20. Thin-layer chromatography of 1. PS-1; 2. PS-4 12 months after preparation; 3. PS-3; 4. PS-2; 5. tristearin. Samples 1, 3 and 4 were run at the time of preparation.

TABLE IV

NMR LINEWIDTHS USING THE PS-2 PREPARATION

% PS in the mixture	$N(CH_3)_3$	$-CH_2-$	$-CH_3$
0	4.9	19.3	19
10	5.3	25.0	18
20	4.5	19.5	21
30	4.8	19.7	19.5*
40	5.0 5.2	21.0 19.7	20 17
100	--- ---	19.0-19.7 19.2	21-22* 22.5*
10+Ca^{++}	6.2	29.0	20*
20+Ca^{++}	5.9	25*	21*
30+Ca^{++}	7.0	31.0	22.5*

Samples were sonicated, Ca^{++} added where indicated and these samples resonicated. Values are from single experiments.

* approximate values difficult to measure

still clearly visible in the increased linewidth after Ca^{++} addition.

Possible reasons for the discrepancies between these and earlier results were considered and these are enumerated below:

1. Purity of the PS preparations

The most obvious possible factor was the homogeneity of the preparations of PS. A third preparation of PS (PS-3) was carried out. In this preparation, the column volume for DEAE-cellulose acetate chromatography was increased by at least 3 times over that described in the method of Sanders (1967), while the elution volumes were not increased. The column volumes and elution volumes used in each preparation are shown in Table V. No major contaminant could be detected by TLC using a conventional chloroform-methanol-ammonia solvent system (Figure 20) but material running with triglyceride could be seen when petroleum ether-diethyl ether-acetic acid was used as solvent. This impure PS-3 gave single peaks for the $-CH_2-$ resonance in NMR spectra of mixtures containing 10, 30, 40, and 100% PS.

2. Autoxidation of phosphatidyl serine

Another possible source of the discrepancy of results is autoxidation of the PS-1. A sample of PS-3 was exposed to air at room temperature for 89 hours. It was then swollen in water and lyophilized as described in Materials and Methods. A single peak for

TABLE V

PS PURIFICATION BY
DEAE-CELLULOSE ACETATE CHROMATOGRAPHY

Preparation	Column Volume (ml)	Elution volume (ml) 1	2	3	4
Sanders	50	800	400	100	500
PS-1	165	1700	1000	240	2100
PS-2	95	1300	700	200	900
PS-3	155	860	400	100	1350
PS-4	120	1100	600	150	1400

---

1. chloroform: methanol 7:3 v/v
2. methanol
3. acetic acid: chloroform 1:9 v/v
4. acetic acid: chloroform 6:1 v/v (eluting solvent)

ref. Sanders (1967)

the $-CH_2-$ resonance was observed.

It was still conceivable that the fatty acid composition of the PS-1 might have been different from that of subsequent PS preparations. Fatty acid analyses of all PS preparations were therefore performed. The results are shown in Table VI in comparison with those of other workers. The GLC analysis of the fatty acid methyl esters of PS-1 (as the sodium salt), performed approximately one month after its preparation was not carried far enough to detect the presence of any 22:6 (22 carbon atoms long with 6 double bonds) acid or later-eluting components. Fifteen months after preparation, fatty acid analysis revealed 34 % of the total weight of fatty acid emerging in one peak eluting beyond 22:6. This material was also present in PS-3, though in smaller amounts, while none was found in PS-2. When PS-3 was purified by preparative TLC to separate the "triglyceride" impurity and was then analyzed by GLC, this peak appeared as approximately 80% of the fatty acid in both the purified PS and the "triglyceride" impurity.

Since oxidation of lipids is known to be facilitated during thin-layer chromatography, it was thought that the peak might be due to oxidation of the PS. However, storage of PS-2 under nitrogen at - 20°C for one year did not cause an increase in this component, though the weight % of 22:6 had decreased from 11.5 to 7.5%. The oxidation must therefore have been due to

TABLE VI

FATTY ACID ANALYSIS OF PHOSPHATIDYL SERINE PREPARATIONS FROM BOVINE BRAIN WHITE MATTER BY VARIOUS WORKERS

Fatty acid*	1	2	3	4	5	6	7	8	9	10	11	12	13	14
12:0	0.11	0.8												
12:1		0.6												
13:0	0.13													
14:0	0.37	0.5					0.1	1.0	0.2	0.3	0.2	0.1	0.2	0.9
14:1	0.14	0.4												
15:0	0.14													
15:1	0.14													
16:0	1.22	3.3	3.2	2.7	2.5	---	1.2	1.9	2.1	1.3	1.0	0.8	28.4	32.0
16:1	0.36	0.4												
16:2		0.2												
17:0	0.47						0.3	0.8	0.4	0.2	0.3	0.2		
17:1	0.12													
18:0	49.9	48.3	32.7	39.2	57.4	48.9	42.5	37.7	54.4	23.5	30.0	32.2	13.6	12.3
18:1	30.3	29.4		32.4	34.4	37.2	36.4	31.5	31.6	19.8	25.5	27.6	31.0	32.0
18:2	7	2.4	51.6			---							14.4	15.7
18:3		1.4					3.9	4.5	3.9	3.5	3.7	3.7		0.4
20:1	4.58	3.1		3.9	4.6	4.8								
20:2	1.22	2.1	7.1				0.4	1.4	0.4	0.7	1.0	0.5	6.2	1.9
20:3	0.46	t							0.4	0.7	0.7			
20:4	0.19			5.7	---	4.0		3.6	1.0					
20:5	---						2.2	1.4		3.5	3.0	3.9		
22:3	0.84		5.4	2.4?	1.2			2.5	4.3					
22:4	0.46			2.4?	---		0.9	2.5		1.9	2.3	2.2		
22:5	0.25							2.0	0.8	1.1	2.1	0.9		
22:6	0.92			11.2	---	3.8	11.5	7.5	ND	7.4	10.2	9.6	3.8	2.0
19:0	0.28													
19:1	0.62													
20:0	0.72	2.7						0.5	0.2		0.3			
22:0	1.14	3.0												
22:1	0.88	1.1												
22:2	1.22													
24:0							0.8							
unidentified								0.1	ND	34.2	17.7	10.1		

1. Silver et al. (1963)
2. Graham and Lea (1972)
3. Klenk and Böhm (1951)
4. Zitko et al. (1970)-fresh sample
5. Zitko et al. (1970)-after 4 months storage under N_2 at -14°C
6. Papahadjopoulos and Miller (1967)
7. PS-2 fresh
8. PS-2 twelve months after preparation (isothermal at 220°C: poor separation in earliest part of spectrum)
9. PS-1 one month after preparation (Na salt) (isothermal at ≈160°C: poor separation in earliest part of spectrum).
10. PS-1 fifteen months after preparation
11. PS-3 three weeks after preparation
12. PS-3 one month after preparation
13. PC one month after preparation
14. PC 23.5 months after preparation

Fatty acids are designated by the number of carbon atoms followed by the number of double bonds

* total unsaturated of that chain length
t trace
* total eluting between 20:1 and 20:4
* total eluting between 20:4 and 22:6
= 1.4% is total for 20:4 and 22:1
= 1.0% is total for 20:4 and 22:1
= 0.8% is total for 22:5 and 24:0

an acetic acid-catalyzed reaction during the preparation procedures. There is less danger of autoxidation in PC preparations since PC contains fewer polyunsaturated fatty acids.

The unknown component of the GLC analysis may represent a mixture of compounds. Hansen and Smith (1966) found that there occurred formation of methoxy compounds from oxidized lipids isolated from sheep perinephric fat when similar esterification conditions were used. The various products appeared in the GLC as one peak with a trailing edge.

3. Effect of sonication on physical structure

The efficiency of sonication is difficult to control so that breakdown of large multilamellar structures may be more or less complete. In addition, the rate and extent of vesicle aggregation after sonication may vary. Phospholipid molecules in larger vesicles could experience a different environment from those in smaller vesicles. A mixture of structures would give rise to multiple peaks. PS and PC might segregate preferentially into one or other of these structures. It must be stated that this explanation is unlikely since splitting of resonance lines correlated well with the PS-1 preparation and was not observed with any previous or subsequent PS preparation.

4. Effect of sonication on chemical structure

Chemical degradation, especially hydrolysis to monoacyl PS, may have occurred in PS-1, PS-2 or PS-3. Some

breakdown did take place as judged by TLC (Figure 21). The amount of products was not quantitated. Hauser (1971) stated that he had found conditions to eliminate degradation of PC during sonication but Hauser and Phillips (1973) admitted that approximately 3-4% degradation occurred during sonication of PS.

5. Effect of contaminating paramagnetic ions on linewidths

In these experiments, the Varian HA-100 instrument was operated at the highest gain setting and at the limit of its sensitivity. The presence of trace amounts of paramagnetic ions, for example manganese, copper, or zinc, could lead to enough broadening to effectively obliterate any separation of peak resonances. Radda (1974) has suggested that phospholipid preparations can be contaminated with such ions. The addition of EDTA removes paramagnetic ions (Berden et al. 1975). However, the presence of paramagnetic ions should primarily affect the head groups of the phospholipid. It would therefore be necessary to postulate that all the paramagnetic ions bound to PS and that the $-\overset{+}{N}(CH_3)_3$ of the PC was not affected. This is rather unlikely.

Figure 21. $CHCl_3$: CH_3OH: 6N NH_4OH thin layer chromatography of

1. a chloroform solution of 20% PS- 80% PC (6.0 mg PS, 24.0 mg PC) before sonication
2. the same 20% PS- 80% PC sample after 25 min. sonication (from the D_2O suspension)
3. PS in chloroform
4. PC in chloroform

CHAPTER V

DISCUSSION

Structure and properties of Ca^{++} complexes

Water molecules surrounding a Ca^{++} ion can be regarded as small ligands which form moderately strong co-ordination complexes with the central ion. Thus, the hydration shell forming the immediate environment of the metal ion (inner sphere of hydration) possesses the same characteristics of co-ordination number and spatial arrangement as any other ligand of comparable size and polarizability. The molecules of the inner sphere are rather immobile and cannot easily exchange with other solute molecules because of the relatively tight binding. Calculations for $Ca^{++} \cdot H_2O$ give a free energy of 55 kcal/mole and a Ca-O distance of 2.40 Å for binding of the first water molecule. In addition to the first hydration sphere, there is a surrounding region of water, the outer hydration sphere or B region which has properties different from those of the bulk aqueous phase. The molecules in the outer sphere can exchange somewhat more readily than those of the inner sphere.

For the series Mg-Ca-Ba, an increasing ionic radius is associated with a decreasing thickness of the total sphere of hydration. In other words, the smaller the cation itself the greater is its influence on the surrounding water. This is an observation of profound

importance in selection of an appropriate cation for a particular biological function. In aqueous solution, the formation of a complex with a ligand other than water involves the substitution of a water molecule by molecules of the ligand. In principle, further substitutions may occur on the same Ca^{++} ion to a maximum equal to the preferred co-ordination number of eight. In practice however, the number of replacements with large ligands is unlikely to exceed four, due to steric considerations.

Ca^{++} binds preferentially to phosphate groups in an environment also containing carboxyl groups and amino groups. However, the two last groups can stabilize binding by phosphate through the formation of mixed chelates as in phosphorylserine and phosphorylated peptides and proteins. Here again steric considerations will be important.

The crystal structures of a few representative metal-ligand complexes are known. Binding of Ca^{++} to phosphate groups is illustrated by the crystal structure of calcium 1-naphthylphosphate trihydrate and calcium thymidylate. In the former, oxygen atoms are contributed from four separate phosphate groups and the Ca-O distances are from 2.355 Å to 2.441 Å whereas in the latter more than one oxygen atom is contributed from the same phosphate group and this leads to Ca-O distances from 2.29 Å to 2.65 Å. In calcium dioxyacetate, the crystal structure consists of $CaO(CH_2COO^-)_2 . 5\ H_2O$ complexes, linked by H-bonding involving

one of the water molecules, and the co-ordination is eight-fold, consisting of five water molecules, two carboxyl groups and the ether oxygen of the tridentate ligand (Uchtman and Oertel 1973). In this and other carboxyl complexes the Ca-O distances are all within 2.4 Å to 2.5 Å. The trans-trans skeletal C-C-O-C-C conformation is induced by Ca^{++} binding, since the oxydiacetate in the absence of Ca^{++} exists as a mixture of rotational isomers.

Ca^{++}-lipid complexes

The interaction of Ca^{++} ions with PS depends not only on the chemical nature of the functional groups available (phosphate, carboxyl, amino) but also on their mode of assembly as determined by the physical structure of the lipid-water system. Thus, the reactions of ligands located in the lipid-water interface differ sharply from those of comparable ligands dissolved in a bulk aqueous solution. Association constants have been determined by a number of investigators using different methods and these are collected in Table VII. For comparison, constants for several water-soluble ligands are shown. Based on some of these results, Hendrickson and Fullington (1965) suggested a mixed chelate structure utilizing phosphate, carboxyl and amino groups (Figure 22). It is also apparent that the values for Ca^{++}-lipid complexes are much higher than those for soluble Ca^{++} salts. In most cases, the electrostatic energy due to the field derived from the assembly

TABLE VII

ASSOCIATION CONSTANTS (K_a) FOR Ca^{++}-LIPID COMPLEXES AND COMPARISON WITH MODEL COMPOUNDS

Compound	Method	log K_a	Reference
PS	Titration	4.03	Hendrickson and Fullington 1965
PS	Microelectrophoresis	4.07	Barton 1965
PS	Surface Balance	7.15[a]	Hauser et al. 1969
CH_3COOH	Titration	1.00	Phillips and Williams 1966
$(CH_2COOH)_2$	Titration	2.00	"
$CH_2(COOH)_2$	Titration	2.49	"
$(COOH)_2$	Titration	3.00	"
$CH_2(NH_2)COOH$	Titration	1.43	"
$CH_3CH(NH_2)COOH$	Titration	1.24	"
$HOOCCH_2NHCOCH_2NH_2$	Titration	1.24	"
$H_2PO_4CH_2CH(NH_2)COOH$	Titration	3.25(ML)[b] 2.50(MHL)[c]	Hendrickson and Fullington 1965
$H_2PO_4CH_2CH_2NH_2$	Titration	2.00(ML)[b] 1.35(MHL)[c]	"
Glycerylphosphoryl inositol diphosphate	Titration	3.27(ML)[b] 2.22(MHL)[c]	"

[a] includes electrostatic free energy term contributing approximately 3.0 to log K_a

[b] Ca^{++} liganded to completely deprotonated phosphate group

[c] Ca^{++} liganded to monoprotonated phosphate group

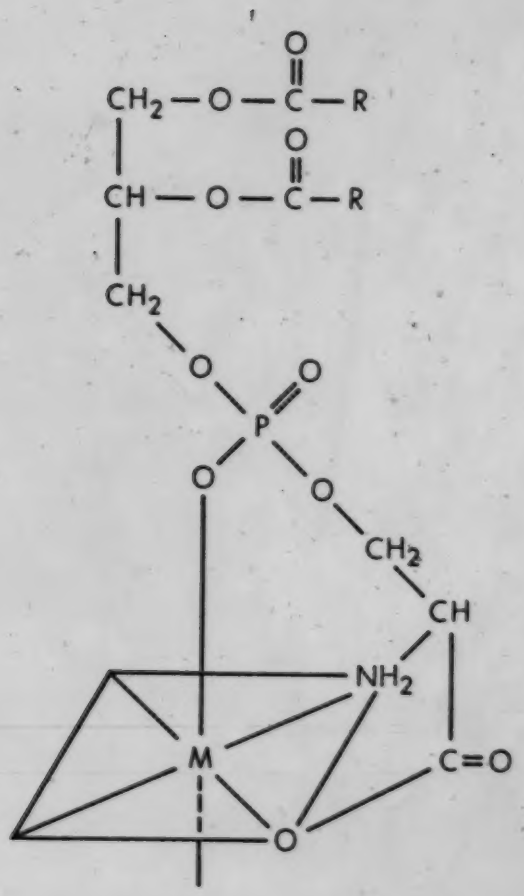


Figure 22. Possible structure for a phosphatidyl serine-metal complex (according to Hendrikson and Fullington 1965).

of fixed surface charges has been subtracted from the total interaction energy in computing the PS-Ca^{++} association constants, so that the avidity of the lipid ligands for Ca^{++} must be due to their lattice array. Papahadjopoulos (1968) demonstrated that binding of Ca^{++} to PS monolayers gave rise to a decrease in surface pressure and suggested the formation of a linear polymeric lattice structure (Figure 23), based on the chelating unit suggested by Hendrickson and Fullington (1965).

Just as Ca^{++} binding induces conformational changes in small molecules, so the structure of PS bilayers may be modified by the interaction with Ca^{++}. The studies reported in this thesis, as well as those of other workers, show that such modification does occur. Thus, the increases in linewidth of the $-CH_3$, $-CH_2-$ and $-\overset{+}{N}(CH_3)_3$ resonances or the broadening of the downfield $-CH_3$ and $-CH_2-$ components observed after addition of Ca^{++} to mixed PS:PC bilayers, can be interpreted as being due, in part, to a 'condensation' effect, equivalent to that observed in monolayers (Rojas and Tobias 1965, Hauser et al. 1969). A second effect which can contribute to line broadening may occur through reduction of the electrostatic component of the energy barrier to particle collision. Indeed visual inspection indicated that addition of Ca^{++} to PS-rich lipid mixtures followed by sonication increased light scattering considerably, consistent with particle aggregation. Papahadjopoulos and Watkins (1967) noted that elevated Ca^{++}

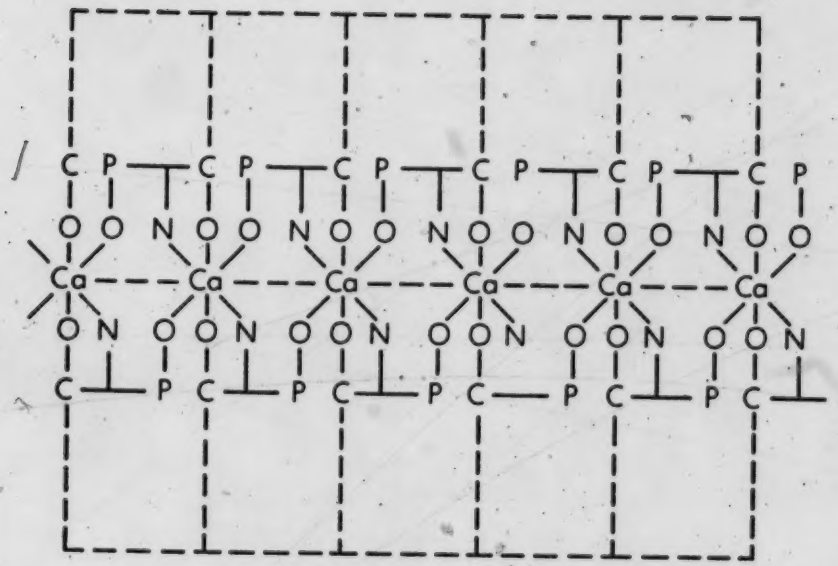


Figure 23. The linear polymeric lattice structure for a phosphatidyl serine-metal complex suggested by Papahadjopoulos (1968).

concentrations increased the rate of self-diffusion of K^+ through PS-containing bilayers. Later studies with PS bilayer films showed that Ca^{++} stabilized and increased the resistance of the membrane when symmetrically disposed but led to destabilization when present on one side only (Papahadjopoulos and Ohki 1969, Ohki 1972). Papahadjopoulos et al. (1975) reported that cochleate cylinders ("Swiss rolls") were formed when Ca^{++} was added to the outside of sonicated PS vesicles. Under the conditions reported here, where sonication followed Ca^{++} addition, it was expected that stable bilayers would be formed. Therefore, particle aggregation due to neutralization of the electrical barrier was probably not followed by particle fusion. Nonetheless, aggregation should lead to a decreased tumbling rate. It is not known whether this decrease would contribute significantly to line broadening (see Chapter I).

Phase separations in mixed lipid bilayers

There is considerable evidence to indicate that phase separations can occur in bilayers composed of two or more lipid species. Phillips et al. (1972) showed that mixtures of distearoyl- and dioleoyl-PC produced two thermal transitions by DSC. Similar results were obtained by Oldfield et al. (1972) with mixtures of dibehenoyl- and dioleoyl PC. In addition to lateral separations resulting from differences in fatty acid composition, transversely asymmetric distributions can occur in mixtures of lipids having different polar head groups. Michaelson et al. (1973)

used the separation of ^{31}P NMR resonances of PC and PG to demonstrate that PG is preferentially located in the outer half of the bilayer in mixed PC:PG vesicles. This asymmetry may originate in charge and size differences between the two head groups (Berden et al. 1975). In mixtures of PC with PA, PE, PI, or PS, the lecithin appeared to be located preferentially in the outer half of the bilayer while in PC:sphingomyelin mixtures the converse was true (Berden et al. 1975). Asymmetric distribution of lipids in mammalian erythrocyte membranes has been demonstrated with chemical and enzymatic probes (Bretscher 1972) but the evidence for this is not unequivocal.

Structural effects of Ca^{++} on PS:PC mixtures have been studied by Ohnishi and Ito (1973, 1974) using a nitroxide spin label attached covalently to C5 of the 2-acyl substituent of PC. When low concentrations of spin labelled PC (PC*) were mixed with beef brain PS, no exchange broadening and no anisotropy were observed. This indicated that there was a uniform distribution of PC* in a fluid bilayer. The spectrum of PC* mixed with egg PC showed anisotropy, reflecting the closer packing and greater order of the more saturated lecithin fatty acids. The addition of Ca^{++} to PC vesicles caused no change in the ESR spectrum. When Ca^{++} was added to PS vesicles, the spectrum was broadened, owing to intermolecular spin-spin exchange interaction.

The authors concluded that the PC molecules, which had previously been uniformly distributed in the PS, separated from the PS environment to form clusters. The broadening caused by this separation was less than that seen in pure PC* vesicles, implying some contamination of this PC* cluster with PS molecules. Conversely, the broadening of minor peaks and the presence of shoulders in the ESR spectrum demonstrated the presence of some PC* molecules in regions where the motional freedom is restricted, that is in the PS region.

In interpreting this data, one must remember that it is the spin labelled lipid that is observed rather than unmodified lipid. The presence of the bulky nitroxide group enhances the partitioning of the molecule into the more disordered phase. It has been shown that spin labelled lipid molecules migrate preferentially to the fluid regions of the bilayer (Keith and Mehlhorn 1972, Oldfield et al. 1972). We thus have no information on the behaviour of unlabelled PC and we cannot conclude that the PC is uniformly distributed in the PS, in the absence of Ca^{++}. There is no contradiction, therefore, with the suggestion in Chapter III that a PS-rich phase can exist in equilibrium with a mixed PS:PC phase.

The results of Ohnishi and Ito (1973, 1974) suggest that some PC* remains in the PS-rich phase even after addition of Ca^{++}. Deletion of the bulky nitroxide residue should enhance inclusion of PC in the ordered PS

matrix. Such a deduction would be in accord with our data in Chapter III. Our results showing a broadening of the $-\overset{+}{N}(CH_3)_3$ peak of PC on addition of Ca^{++} to mixed PS:PC vesicles can best be explained by the intercalation of PC molecules in a PS-Ca^{++} matrix.

Papahadjopoulos et al. (1974) and Jacobson and Papahadjopoulos (1975), using DSC, have obtained evidence for phase separations in bilayers after addition of Ca^{++}. A mixture of 66% beef brain PS-34% dipalmitoyl PC in 0.1 M NaCl at pH 7.4 showed only a broad thermal transition from 5°C to 28°C. In the presence of 0.01 M Ca^{++}, two broad transitions centered at 28° to 29° C and 42° C were observed. The higher value corresponds to the transition temperature of pure dipalmitoyl PC, implying that it is now clustered. The lower temperature would represent the PS-Ca^{++} transition. However, pure PS-Ca^{++} showed a transition at 18°C, at Ca^{++} concentrations below 1 mM, and the transition is abolished at higher Ca^{++} concentrations. Thus, the authors concluded that the PS-Ca^{++} phase "still contains considerable amounts" of dipalmitoyl PC.

A mixture containing 66% PS and 34% distearoyl PC showed similar behaviour. In the absence of Ca^{++}, there was a broad transition at 42° C. With Ca^{++}, the major transition was at 53-55°C with a broad transition at a lower temperature. Distearoyl PC has a transition temperature of 55.5°C. No separation occurs, however, if the Ca^{++} concentration is lower (5 mM) or if the PC is 50%

or more of the phospholipid. Also, at pH 8, but not at pH 6, dipalmitoyl PC separated from a mixture of 66% dipalmitoyl PA and 34% dipalmitoyl PC in the presence of Ca^{++}.

Ca^{++}-protein complexes

The ability of Ca^{++} to bind to prothrombin was first inferred from the work of Ganrot and Nilehn (1968) who noted a reduced electrophoretic mobility of the protein on agarose gel after addition of Ca^{++} salts to the electrophoresis buffer. By quantitative immunoprecipitation, Stenflo and Ganrot (1973) demonstrated a conformational change in the protein on binding of Ca^{++}. Bull (1973) noted a similar effect using sedimentation-velocity ultracentrifugal analysis.

As was discussed at greater length in Chapter I, the Ca^{++} binding sites of prothrombin reside in the N-terminal (Fragment 1) region of the polypeptide. It seems likely that the malonyl groups of γ-carboxyglutamyl residues constitute the principal ligands. The value for log K_a of 2.49 for malonic acid (Table VII) is in good agreement with that for the lower affinity sites (log K_a 2.54) on prothrombin determined by Hui and Barton (Barton 1971). However, the values reported by Benson et al. (1973) (log K_a 3.2) and the values for the high affinity sites (log K_a 4) observed by Hui and Barton (Barton 1971) can only be explained by assuming the intervention of additional ligands. The most likely candidates for such interaction

are carboxyl groups of other glutamyl or γ-carboxyglutamyl residues. Presumably, the formation of such tridentate or quadridentate complexes would have to be a function of the tertiary structure of the protein. In agreement with this, modification of prothrombin with 8 M urea plus reduction of disulphide bonds caused some reduction in total Ca^{++} binding but did not abolish it (Nelsestuen and Suttie 1972a). It is possible that the binding of the first one or two Ca^{++} ions to γ-carboxyglutamyl residues of prothrombin causes a conformational change which brings other carboxyl residues into closer proximity with the metal ion. Crystallographic analysis of the metal-free and metal-containing protein will be required to confirm or refute these suggestions.

Lipid-Ca^{++}-protein complexes

Initially, the formation of ternary complexes will depend upon the relative association constants of the contributing binary complexes. As indicated above, both free phosphorylserine and malonic acid bind Ca^{++} with log K_a close to 2.5, whereas the same groups present in PS and prothrombin respectively bind with log K_a near 4.0, due to multiple liganding effects. Maximum formation of lipid-Ca^{++}-prothrombin complexes was observed to occur when Ca^{++} concentration was between 0.01 M and 0.025 M, decreasing beyond these limits. Under these conditions ternary complex formation could occur only if log K_a is equal to or exceeds log K_a for lipid-Ca^{++} and for protein-

Ca^{++} complexes. This places the value of log K_a for ternary complex formation in excess of 4.0. The fact that the complex is disrupted by EDTA (Bull et al. 1972) places an upper limit of 10.6 on log K_a. A graded series of chelating agents, with log K_a for metal complexation in the range from 4.0 to 10.6, could be tested for ability to disrupt the complex and hence provide information on its stability. Clearly, knowledge of the association constant places constraints on proposals for the structure of the complex. Figure 24 shows a schematic representation of a possible structural unit linking lipid and protein.

It seems unlikely that ternary complex formation induces significant structural alterations in the lipid bilayer, over and above those caused by Ca^{++} alone. Thus, the linewidths of the $-\overset{+}{N}(CH_3)_3$, $-CH_2-$ and $-CH_3$ signals or the relative intensities of the $-CH_2-$ and $-CH_3$ components were neither significantly increased nor decreased by addition of prothrombin to Ca^{++}-lipid mixtures. These findings infer, but do not prove, the absence of apolar interactions and hydrophobic effects involved in lipid-protein interaction (see below). Whether any conformational change occurs in the protein component as a result of complex formation cannot be determined at this time.

Other model systems

The complex of glycyl-glycine-Ca^{++}-PS reported by Bulkin and Hauser (1973) and that of wheat flour protein-Ca^{++}-PS reported by Fullington (1967) appear to have similar

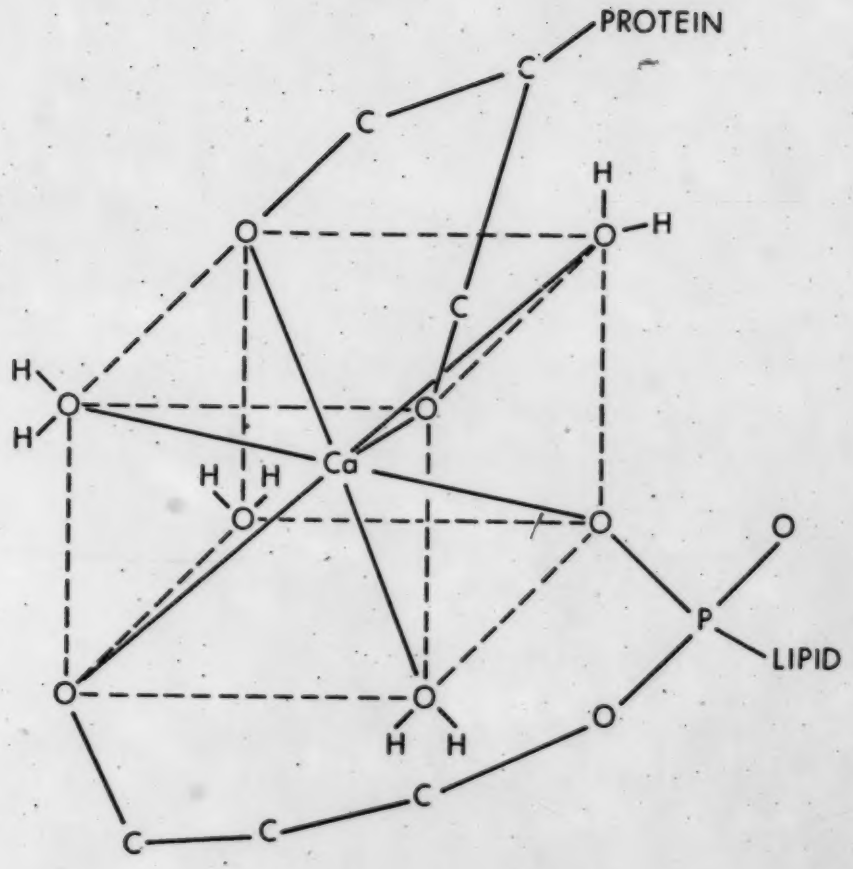


Figure 24. Schematic representation of a possible structural unit linking lipid and protein. Diagram illustrates maximum eight-fold co-ordination of calcium with approximately equal bond angles and ligand distances. (not to scale).

formation and structural characteristics to the above. In contrast, the interaction of the brain-specific protein S-100 with Ca^{++} and PS appears to be quite different in that Ca^{++} induces a conformational change in the protein exposing hydrophobic groups capable of interacting with the lipid.

The interaction of insulin with acidic phospholipids at pH 3.5 is mostly dependent on ionic effects (Perry et al. 1971). The protein is polybasic at this pH. X-ray diffraction studies on multibilayer systems showed that insulin enters and replaces the water layers between adjacent lipid layers (Rand and SenGupta 1972). Therefore, insulin appears to bind at the surface of the bilayer. ESR spectra of a cholestane spin probe incorporated into multibilayers of ganglioside-depleted brain lipids showed that insulin, ribonuclease, lysozyme and trypsin oriented the spin axis of the probe perpendicular to the bilayer (Butler et al. 1973), an effect which was mainly eliminated by 0.15 M NaCl. Bovine serum albumin, β-lactoglobulin and cytochrome c each induced a more random orientation of the spin axis either in the presence or absence of salt. Such complexes are presumably additionaly stabilized by non-ionic forces.

In contrast with these results, Van and Griffiths (1975), using nitroxide derivatives of stearic acid or cholestane, did not find any effect of cytochrome c on orientational anisotropy, on the polarity of the probe environment or on the fluidity gradient. However, lysozyme,

cytochrome c and human serum albumin all increased the Na^+ permeability coefficient of PS vesicles and increased the surface pressure of PS monolayers (Kimelberg and Papahadjopoulos 1971, 1971a). Increasing ionic strength reversed the effect of cytochrome c on permeability but not that of lysozyme. Therefore, the relative contribution of electrostatic to hydrophobic effects for complex stabilization is much greater for cytochrome c than for lysozyme. The extent of broadening of the $-\overset{+}{N}(CH_3)_3$ resonance in Figure 12 indicates that the molecular motion of the choline group is restricted by the proximity of the protein adsorbed at the surface of the bilayer. The broadening of the downfield $-CH_2-$ component (Figure 14) could be the result of this immobilization being transmitted down from the choline group or it could be due to hydrophobic interactions.

Hydrophobic interactions between PC and D-β-hydroxybutyrate dehydrogenase were inferred from the fact that the complex was stable in the presence of 0.8 M NaCl, and that inclusion of either cholesterol-dicetyl phosphate (negatively charged) or cholesterol-stearylamine (positively charged) in the PC did not disrupt the complex (Hexter and Goldman). NMR revealed that both the choline and hydrocarbon chain regions of lysolecithin are involved in the binding of lysolecithin to this enzyme and to malate dehydrogenase (Dodd 1972). This interaction of

malate dehydrogenase with lysolecithin stabilizes its enzymatic activity. NMR also indicated hydrophobic interactions between glutamate dehydrogenase and PS or cardiolipin while the ionic strength behaviour of the interaction showed it to be primarily electrostatic.

When casein or casein fractions were added to a 1% lysolecithin solution, hydrophobic interactions were observed: the NMR $-CH_2-$ signal from lysolecithin was broadened while the $-\overset{+}{N}(CH_3)_3$ resonance was not (Barratt and Rayner 1972). Electron microscopy, infrared and fluorescence CD spectroscopy (Crane 1972) indicated that cytochrome oxidase interacts with the hydrocarbon region of the phospholipid bilayer. This is supported by the abolition of spectral anisotropy and fluidity gradients as measured with ESR probes (Jost et al. 1973). Hydrophobic interactions are thus a well established feature of some lipid-protein complexes.

Glucose-6-phosphatase from rat liver, 90% freed from associated lipid was not reactivated by dipalmitoyl PC. Reactivation with monounsaturated PC required addition of detergent while sonication of dioleoyl PC was sufficient. This indicates that complex formation is favoured by the presence of liquid-crystalline lipid phases.

Biological significance of prothrombin-Ca^{++}-lipid complexes

Circulating platelets carry on their surface substantial amounts of adsorbed clotting factors including

prothrombin, and Factors V, VIII, IX, and X, as demonstrated by clotting tests utilizing specifically deficient substrate plasmas (Jevons and Barton 1971, Walsh 1972, Miale and Kent 1975). Some understanding of the mechanism of prothrombin activation at the platelet surface has emerged from studies on model systems (Barton and Hanahan 1969, Jackson et al. 1974).

The primary event involves proteolytic cleavage of prothrombin by the serine protease, activated Factor X. The reaction is greatly potentiated by Factor V. Binding of prothrombin to lipid surfaces requires Ca^{++} and is reversed by EDTA and low pH (Bull et al. 1972, Barton et al. 1970). The characteristics of binding are similar to those of periplasmic or extrinsic membrane proteins (Heppel 1971). In contrast, the attachment of Factor V to lipid bilayers is not facilitated by Ca^{++} ions and is not readily reversed (Papahadjopoulos and Hanahan 1964). The protein can be extracted with detergents but only with loss of activity. It appears therefore to have similar characteristics to intrinsic membrane proteins (Singer and Nicholson 1972). Clotting factors involved in other phases of the coagulation sequence can be similarly categorized (Barton 1967). Experiments in which cells were washed with aqueous buffer solutions indicated that the differential binding characteristics of clotting factors are comparable to those in model lipid systems.

Recently, it has been shown that a liquid-crystalline or 'fluid' phase is essential for the catalytic activity of lipid bilayers in blood clotting tests (Sterzing and Barton 1973). This implies that prothrombin conversion is dependent on lateral and rotational diffusion of the reacting protein molecules attached to or embedded in the lipid milieu. The higher microviscosity of a gel phase structure, without necessarily impeding the initial binding of the individual protein components to the bilayer, would greatly reduce the rate of such molecular interactions, resulting in low biological activity. Since Ca^{++}-prothrombin has been shown to induce more 'condensed' or gel-like regions in the lipid bilayer, the sequestering of such regions from the fluid milieu may be required to permit the necessary protein-protein interactions to occur, as illustrated in Figure 25.

Figure 25. Schematic representation of lipid-protein interactions involved in prothrombin activation.

prothrombin molecule;

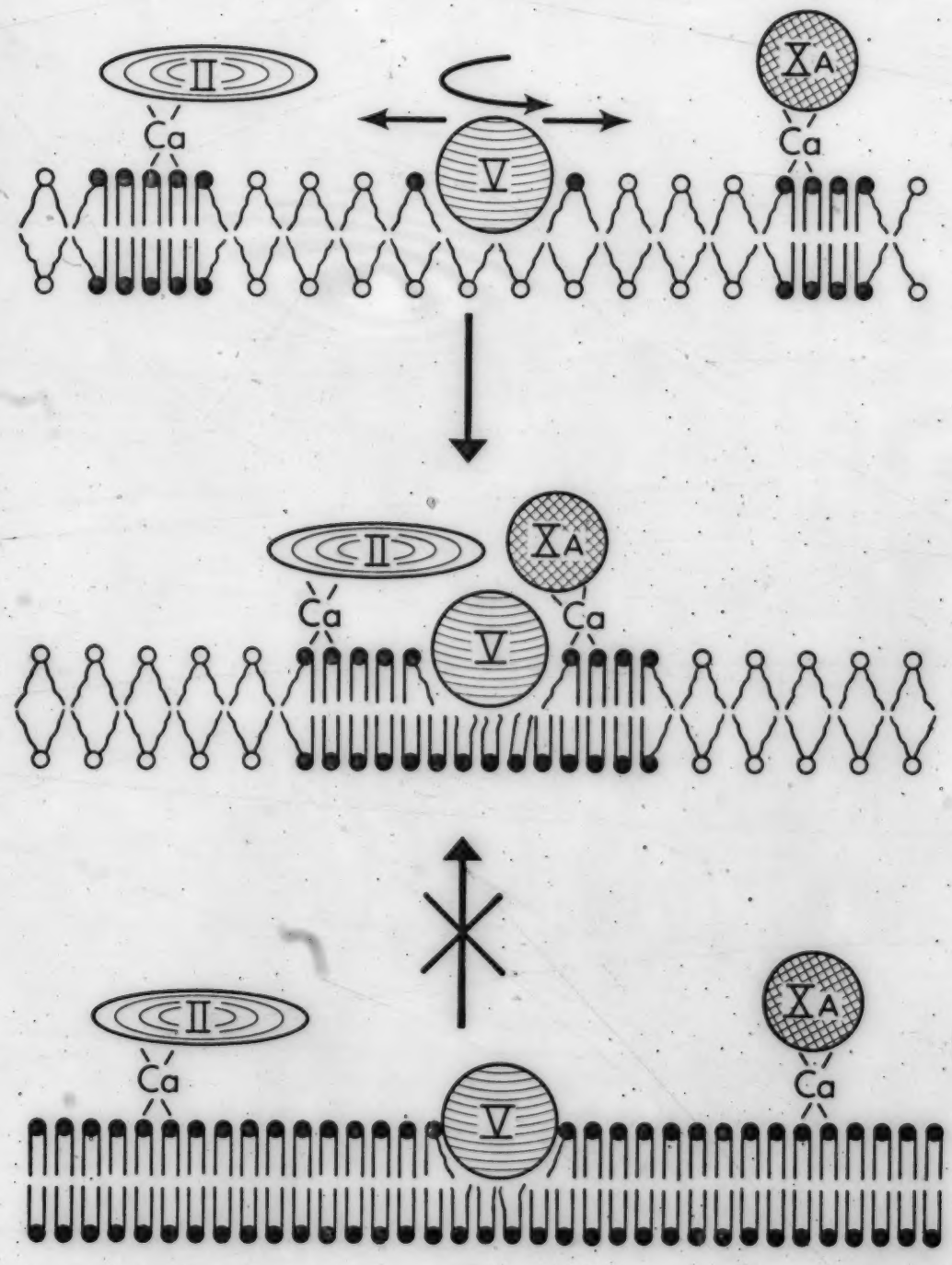
Factor V molecule;

activated Factor X molecule;

lipid molecules in "fluid" or liquid crystalline condition;

lipid molecules in gel condition;

Binding of prothrombin through Ca^{++} bridges induces a localized gel condition but the Factor V molecule is assumed to undergo lateral and rotational diffusion in the fluid regions until complex formation occurs. Complex formation would be kinetically hindered if the lipid were entirely in the gel condition.


II
Ca
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Suggestions for further work

The use of a more powerful proton NMR instrument would increase the separation of the $-CH_2-$ and $-CH_3$ resonances, thus allowing more accurate determination of the linewidths, and would improve the signal to noise ratio. Alternatively, ^{13}C-NMR allows some of the $-CH_2-$ signals to be observed apart from the main $-CH_2-$ envelope. With ^{13}C, it may be possible to study more precisely the presence or absence of hydrophobic effects by utilizing phospholipids of defined chemical structure. In any case, the direct determination of relaxation times from pulse experiments would be advantageous.

Chang and Chan (1974) reported that commercial dipalmitoyl PC, which gave a single spot on TLC, exhibited different behaviour from the same material after purification. They attributed the difference to the presence of a small amount of palmitic acid. In our experiments, the "triglyceride" impurity could not be detected by the usual chloroform: methanol: ammonia or by the chloroform: methanol: acetic acid TLC systems. It would therefore seem advisable to check all phospholipids in both apolar and polar solvent systems.

The large variation in fatty acid compositions of PS given in the literature (Table VII) would suggest that fatty acid analysis of phospholipids be required. It is possible that the beef brain PS may vary from one geographic area to another. In any case, such a measure

would indicate the presence of oxidation products. Contamination or oxidation may explain the discrepancies between the 220 MHz proton NMR spectra presented by Jendrasiak (1972) for egg PC or dioleoyl PC, by Sheetz and Chan (1972) for dipalmitoyl PC and by Darke et al. (1972) and Finer et al. (1971) for egg PC. The first two authors reported a doublet for the $-\overset{+}{N}(CH_3)_3$ resonance while the latter show only a single peak. Bystrov et al. (1971) have shown that addition of Eu^{++} or Mn^{++} to sonicated lecithin vesicles causes two $-\overset{+}{N}(CH_3)_3$ signals to be observed. This interpretation is inapplicable here because any addition of ions preceeded sonication.

PC does undergo some degradation during sonication, according to Hauser (1971). Because of its higher content of poly-unsaturated fatty acids, PS is more susceptible to oxidation and cleavage of double bonds. The use of the bath sonication method of Johnson and Bangham (1969) would be appropriate, if, as Jackson et al. (1974) suggest, no degradation of PS:PC mixtures occurs.

The formation of precipitates when Ca^{++} is added to PS:PC mixtures of high PS content and the major variation in behaviour between 0 and 20% PS would suggest focussing attention on mixtures of low PS content. The use of prothrombin in such investigations is informative because of the known biological significance of the binding. The use of more concentrated preparations and the development of techniques to reduce the problem of the HDO peak in

NMR spectra which have been developed by the Department of Chemistry at this university would obviate the need for lyophilization.

The use of the fragment 1 region of prothrombin or of some smaller Ca^{++}-binding polypeptide derived from it would simplify the system under study. Depending on the molecular weight of the fragments, it may be possible to observe the effect of binding on both the phospholipid and polypeptide.

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